A randomised controlled trial of SFX-01 after Subarachnoid Haemorrhage – the SAS study.

Ardalan Zolnourian[1] ORCID: 0000-0002-0428-179X, Patrick Garland[2] ORCID: 0000-0002-7679-6426, Patrick Holton[1] ORCID: 0000-0003-1548-9649, Mukul Arora[1], Jonathan Rhodes[3], Christopher Uff[4] ORCID: 0000-0001-9787-8001, Tony Birch[5] ORCID: 0000-0002-2328-702X, David Howat[6] ORCID: 0000-0001-7296-4165, Stephen Franklin[6], Ian Galea[2,7,*] ORCID: 0000-0002-1268-5102, Diederik Bulters[1,2,*] ORCID: 0000-0001-9884-9050

[1]Neurosurgery, University Hospital Southampton, Southampton, UK

[2]Clinical Neurosciences, Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, UK

[3]Neuro Intensive Care, Royal Infirmary of Edinburgh, Edinburgh, UK

[4]Neurosurgery, Royal London Hospital, London, UK

[5]Medical Physics, University Hospital Southampton, Southampton, UK

[6]Evgen Pharma, Nether Alderley, UK

[7]Neurology, University Hospital Southampton, Southampton, UK

*shared senior authorship

Corresponding Author:

Diederik Bulters

d.bulters@soton.ac.uk

Words:4389

Tables:1 Figures:6

Abstract

SFX-01 is a novel drug for clinical delivery of sulforaphane (SFN). SFN is a potent nuclear factor erythroid 2-related factor 2 activator, that reduces inflammation and oxidation, improving outcomes after subarachnoid haemorrhage (SAH) in animal models.

This was a multi-centre, double-blind, placebo-controlled, parallel-group randomised clinical trial to evaluate the safety, pharmacokinetics and efficacy of 28 days of SFX-01 300 mg BD in patients aged 18-80 with spontaneous SAH and high blood load on CT. Primary outcomes were 1) safety, 2) plasma and CSF SFN and metabolite levels, 3) vasospasm on transcranial doppler ultrasound. Secondary outcomes included CSF haptoglobin and malondialdehyde and clinical outcome on modified Rankin Scale (mRS) and SAH outcome tool (SAHOT).

105 patients were randomised (54 SFX-01, 51 placebo). There were no differences in adverse events other than nausea (9 SFX-01 (16.7%), 1 placebo (2.0%)).
SFN, SFN-glutathione and SFN-N-Acetyl-Cysteine AUC_{last} were 16.2, 277 and 415 h×ng/ml. Plasma SFN was higher in GSTT1 null individuals (t=2.40, p=0.023). CSF levels were low with many samples below the lower limit of quantification and predicted by the CSF/serum albumin ratio (R2=0.182, p=0.039).

There was no difference in CSF haptoglobin (1.981 95%CI 0.992-3.786, p=0.052) or malondialdehyde (1.12 95%CI 0.7477-1.687, p=0.572), or middle cerebral artery flow velocity (1.04 95%CI 0.903-1.211, p=0.545) or functional outcome (mRS 1.647 95%CI 0.721-3.821, p=0.237, SAHOT 1.082 95%CI 0.464-2.525, p=0.855).

SFX-01 is safe and effective for delivery of SFN in acutely unwell patients. SFN penetrated CSF less than expected and did not reduce large vessel vasospasm or improve outcome.

Trial Registration NCT02614742 clinicaltrials.gov

Key words: Subarachnoid hemorrhage, randomised clinical trial, sulforaphane, Nrf2, haptoglobin, pharmacokinetics

Introduction

Subarachnoid haemorrhage (SAH) is a subtype of stroke that affects younger patients and has worse outcomes than other forms of stroke[1]. There is only one approved medical treatment (nimodipine) and despite this, poor outcome remains common. Even amongst those deemed to have made a good recovery, fatigue, memory impairment and cognitive deficits are frequent, affecting resumption of activities and return to work[2,3].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor. It is a global regulator of detoxifying enzymes [4], and regulates degradation of red blood cells, haemoglobin, haem, and iron through transcriptional upregulation of CD36 [5], haptoglobin[6], hemopexin [7], haem-oxygenase-1 [8], and ferritin[9]. Nrf2 is expressed in the CNS, upregulated after cerebral insults [10], and plays a key role in conditions where inflammation is the hallmark like SAH[11]. In animal SAH models Nrf2 deletion leads to increased inflammation, oxidative stress, cerebral oedema, neuronal death and poor neurological outcome[12,13,14]. In humans, the minor T allele in the single nucleotide polymorphism rs10183914 in the *NRF2* gene is associated with poor outcome after SAH[15].

Nrf2 is regulated by Kelch-like ECH-associated protein 1 (KEAP1) in the cytoplasm which normally binds Nrf2. Oxidative stress leads to KEAP1 releasing Nrf2, which translocates into the nucleus leading to transcription[11]. Sulforaphane (SFN) stabilises Nrf2 through inhibition of ubiquitination activating the Nrf2 pathway. In rodent models of SAH, SFN reduces early brain injury[12], and cerebral vasospasm reducing behavioural deficits, and improving functional outcome[12,16].

SFN is also protective in ischaemic stroke, reducing infarct volume by 30%[17] and improving neurological outcome in preconditioned animals[18]. Delayed cerebral ischaemia (DCI), occurs 3 days to 3 weeks after SAH and also provides a mechanism though which SFN is of benefit, but with a much larger therapeutic window.

SFN has a short half-life rendering it impractical for clinical use[19]. SFX-01 (Evgen Pharma) is a novel agent comprising SFN complexed with α-cyclodextrin. The α-cyclodextrin ring creates a 'scaffold' around the SFN stabilising it. On ingestion, SFN is released, providing an effective method to deliver SFN clinically. Two phase I trials (NCT01948362, NCT02055716) have shown SFX-01 generates good plasma SFN levels with no serious adverse events and very limited side effects.

We therefore designed a randomised controlled trial to test the safety, pharmacokinetics and efficacy of up to 28 days of SFX-01 (300 mg) two times per day in patients within 48 hours of aneurysmal SAH.

Methods

This was a phase II double-blind, placebo-controlled, parallel-group trial in three tertiary neurosciences centres across the United Kingdom. A summary of the protocol has been published[20], and a full version is in Appendix A. The trial was conducted in accordance with the Declaration of Helsinki and met the international criteria for Good Clinical Practice and

was approved by the National Research Ethics Service (Southern Central Hampshire A) and Medicinal Health Care Authority (MHRA), and was registered on clinicaltrials.gov (NCT02614742). Informed consents were obtained from patients or legal representatives. The Consolidated Standards of Reporting Trials (CONSORT) was followed.

Patients and Eligibility Criteria

The inclusion criteria were: (1) radiological evidence of spontaneous aneurysmal SAH, (2) Fisher grade 3 or 4, (3) 18 to 80 years, (4) within 48 hours, (5) aneurysm treatment not ruled out, (6) previously independent, (7) informed consent from the patient, or legal representative within 24 hours of first dose.

Key exclusion criteria included: (1) plasma creatinine ≥2.5mg/dL, (2) bilirubin ≥2 fold upper limit of normal, (3) pregnancy, (4) follow-up not feasible.

Trial Procedures

After admission, identification by the research team and consent, patients were randomised in a 1:1 ratio to active or placebo. The active group received SFX-01 300mg capsules. The placebo group received capsules containing α-cyclodextrin only. This was administered orally or via nasogastric tube twice daily for up to 28 days from ictus. Randomisation was stratified by World Federation of Neurosurgical Societies (WFNS) grade, using sequential pre-numbered treatment packs. Treatment packs were prepared according to a block

balanced randomisation code by a blinded third party. Patients, nurses, clinicians, lab staff and investigators were blinded to allocation; capsules were identical in appearance.

Outcomes

Full details of trial assessments are in the protocol. These include safety, pharmacokinetics, pharmacodynamics, clinical outcome, and imaging data.

Safety

Safety was assessed using treatment-emergent adverse event (TEAE) reporting. TEAEs were recorded throughout the 180 days of participation, coded following the Medical Dictionary for Regulatory Activities, graded for severity, and followed until resolved. All data were captured and reviewed by the independent data safety monitoring board. Safety blood and urine (full blood count, urea and electrolytes, coagulation screen, liver function tests and urine microscopy) were obtained at baseline, post-dose, and on days 7 and 28. Additionally, clinically obtained bloods were monitored until discharge.

Pharmacokinetics

Although excellent plasma SFN levels have been demonstrated with administration of SFN and SFX-01 in healthy volunteers, this was the first study targeting acutely unwell patients. Moreover, despite the extensive literature supporting SFN in neurological disorders, there is only one animal study (none in humans) quantifying brain penetration[21]. Therefore, paired

CSF and plasma samples were obtained 7 days post ictus. CSF was obtained via lumbar puncture (LP), or an external ventricular drain (EVD) when clinically available. In addition, 12 patients with an EVD consented to a pharmacokinetic sub-study to obtain CSF and blood samples 0,1,2,3,4,5 and 6 hours after dosing on day 3 and 7. Sample collection and analysis of SFN and its metabolites (glutathione (SFN-GSH) and N-acetyl cysteine (SFN-NAC)) are detailed in supplemental methods and the validation report is in Appendix B.

Vasospasm

To determine if SFX-01 reduced middle cerebral artery (MCA) flow velocity following SAH, transcranial doppler (TCD) ultrasound was performed on alternate days during the inpatient stay. MCA flow velocities (time average maximum) were measured bilaterally.

Secondary endpoints

Pharmacodynamic analysis of plasma and CSF malondialdehyde for oxidative stress, and serum and CSF haptoglobin for transcriptional activity and haemoglobin-binding capacity upregulation was performed on day 7. In addition, a serum sample was obtained on day 28. CSF and serum samples were obtained from patients with EVDs on alternate days while they were *in situ.* Details of haptoglobin and malondialdehyde quantification are in supplemental methods.

DCI (new focal deficit or reduction in Glasgow Coma Scale ≥2 not explained by other causes), and initiation of hypertensive therapy were assessed daily. Outcome on the modified Rankin

Scale (mRS) was recorded on day 7, discharge and days 28, 90 and 180 after SAH by a trained study nurse. At these visits the extended Glasgow Outcome Scale (GOSE)[22], 36-Item Short Form Health Survey (SF-36)[23], Brain Injury Community Rehabilitation Outcome Scale (BICRO-39)[24], Checklist for Cognitive and Emotional consequences following stroke (CLCE-24)[25] and Subarachnoid Haemorrhage Outcome Tool (SAHOT)[26] were also obtained.

Glutathione S-Transferase (GST) was genotyped by Kompetitive Allele-Specific PCR (KASP) at LGC Genomics for an exploratory analysis to assess the relationship of GSTM1 and GSTT1 status to levels of SFN in plasma and CSF.

Statistical analysis

The statistical analysis plan (SAP) is in appendix C. The safety analysis included any randomised patient who received at least one dose of study medication. All other analyses were performed on the per protocol population (dosed to day 7 post-ictus or more).

The primary endpoint was maximum (highest of all study visits) MCA flow velocity from an Analysis of Variance (ANOVA) model. The maximum MCA flow velocity per timepoint (highest side) was also analysed using a mixed model repeated measures (MMRM) approach.

Serum haptoglobin and plasma malondialdehyde concentrations were analysed using MMRM. CSF concentrations on day 7 were analysed using an analysis of covariance

(ANCOVA) model with two levels for CSF source (EVD or LP) and an interaction between treatment and CSF source.

The proportion of patients with DCI and those receiving hypertensive therapy was analysed using logistic regression. mRS, GOSE and SAHOT were analysed using proportional odds logistic regression.

In further exploratory analyses, variables were tested for normality with the Shapiro-Wilks test, log transformed as necessary and correlation tested with a Pearson or t-test, and Spearman rank or Wilcoxon signed rank where variables remained non-normally distributed. Logistic regression was used for associations with binary outcomes and Tobit regression with the lower limit of quantification (LLOQ) set as left censored for association with SFN concentrations.

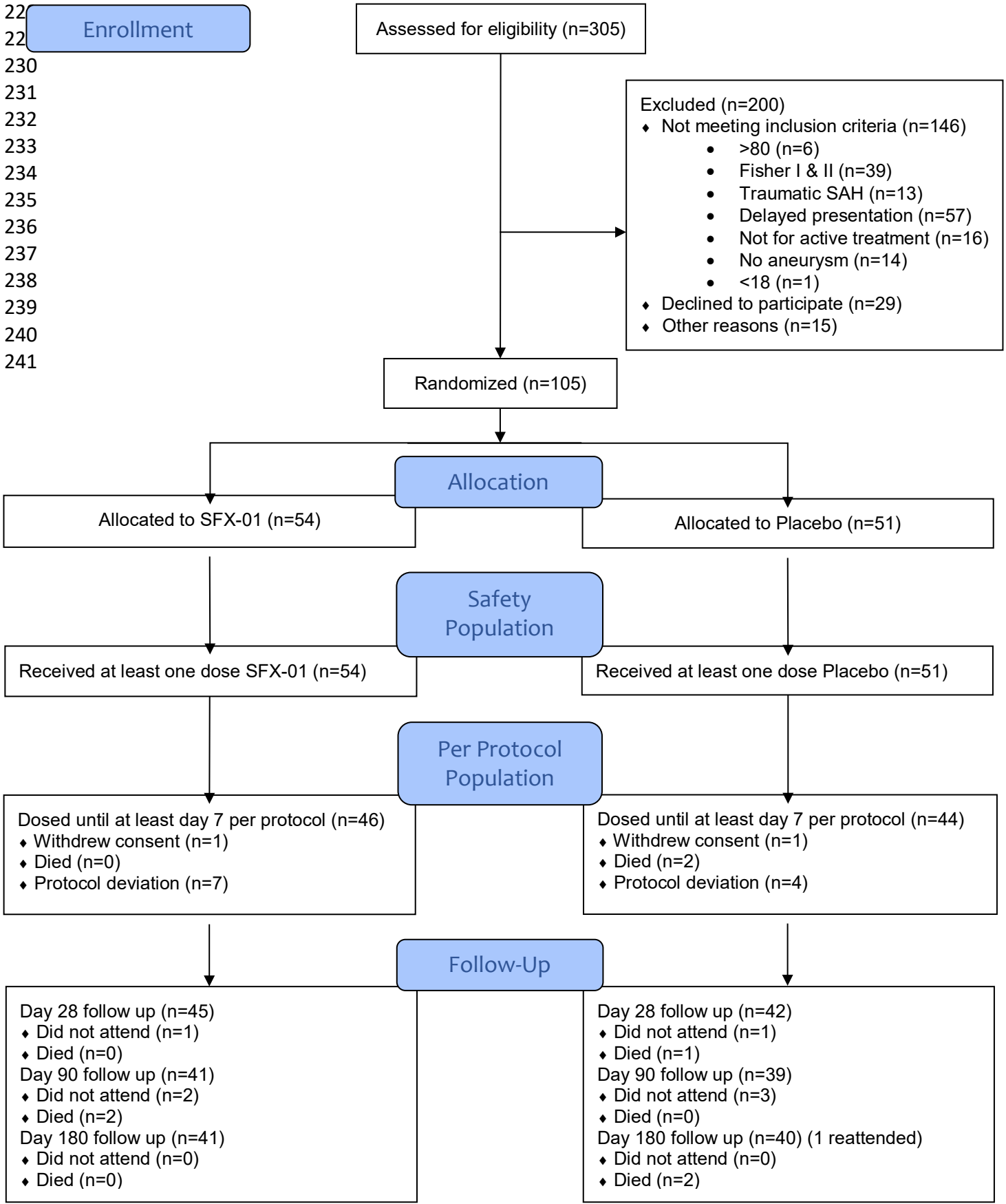
Planned analyses were performed in SAS (SAS Institute, Cary NC) and exploratory analyses in R 4.1.2.

Results

Between April 2016 and February 2019, 305 patients with SAH were screened. 105 patients met inclusion criteria and consented. 54 patients were allocated SFX-01 and 51 placebo. All received at least one dose and are in the intent-to-treat safety analysis. 46 allocated SFX-01

and 44 allocated placebo are in the per protocol analyses (Figure 1). Baseline characteristics are in Table 1.

Figure 1. CONSORT flow diagram of patients screened and recruited to the SAS study



Treatment	SFX-01	Placebo
Age (years), mean (SD)	55.1 (11.9)	55.3 (10.7)
Sex male female	 13 (28.3%) 33 (72.7)	 11 (25%) 33 (75%)
Hypertension	14/46 (30.4%)	13/44 (29.5%)
Fisher Grade 3 4	 16 (34.7%) 30 (65.3%)	 17 (38.6%) 27 (61.4%)
WFNS 1 2 3 4 5	 19 (41.3%) 12 (26%) 0 (0%) 13 (28.3%) 2 (4.4%)	 21 (47.8%) 6 (13.6%) 6 (13.6%) 8 (18.2%) 3 (6.8%)
Aneurysm location Anterior circulation Posterior circulation	 36 (78.2%) 10 (21.8%)	 40 (93%) 3 (7%)
Aneurysm treatment Clip Coil Conservative	 12 (26%) 34 (74%) 0 (0%)	 10 (22.7%) 33 (75%) 1 (2.3%)
Time from ictus to first dose (hrs), mean (SD)	29.1 (12.5)	32.3 (14.4)
Duration of treatment (days), mean (SD)	23.3 (6.0)	23.1 (6.5)

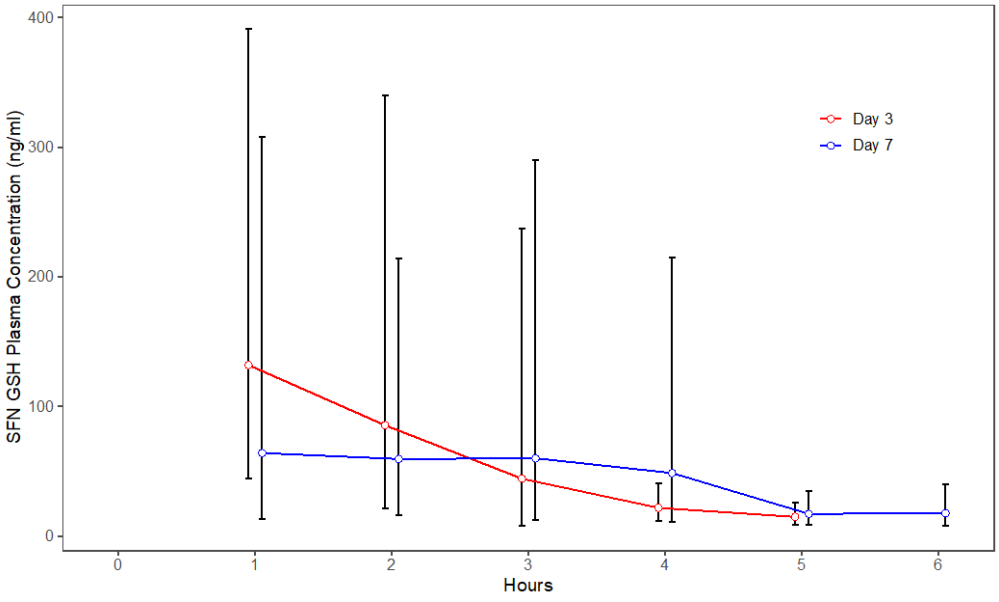
Table 1. Baseline characteristics of the per protocol population.

Fifty-three patients (96.1%) allocated SFX-01 and 48 (94.1%) placebo experienced a TEAE (Table S1). The only difference was nausea in nine (16.7%) receiving SFX-01 and one (2.0%) receiving placebo. Vomiting occurred in five (9.3%) allocated SFX-01 and two allocated placebo (3.9%). Two patients taking SFX-01 and none taking placebo discontinued medication due to nausea or vomiting. There were no differences in haematological or

biochemical parameters at any time (day 1-5, 6-8, 9-14, 15-21, 22-30, >30). There were four deaths in both groups.

Sulforaphane and metabolite plasma and CSF levels

Eight patients in the pharmacokinetic sub-study undergoing hourly plasma and CSF sampling were allocated SFX-01. Plasma SFN-GSH and SFN-NAC are displayed in Figure 2 and Table S2. At every timepoint there was at least one patient with plasma SFN below LLOQ, precluding calculation of a geometric mean as in the SAP.



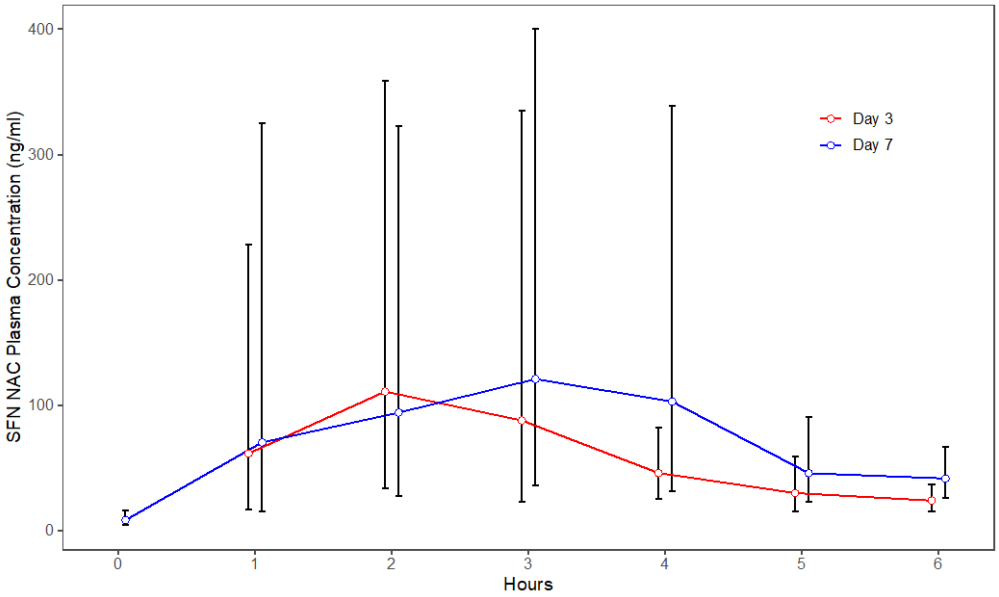


Figure 2. Plasma concentration of a) SFN-GSH and b) SFN-NAC in eight patients in the pharmacokinetic sub-study. Geometric mean ± sd.

There was at least one patient with CSF SFN, SFN-GSH and SFN-NAC below LLOQ at every timepoint, precluding analysis as per SAP. Five of 95 samples taken 1,2,3,4,5 and 6 hours after dosing for SFN on day 3 and 7 were above LLOQ (geometric mean 105 geometric sd 4.41). 5 of 96 samples for SFN-GSH (geometric mean 11.6 geometric sd 1.13), and none of 96 for SFN-NAC were above LLOQ.

Including the eight sub-study patients, 45 of 46 in the per protocol population had a plasma sample taken, and 40 of 46 had a CSF sample taken on day 7. All available plasma and CSF SFN, SFN-GSH and SFN-NAC measurements for patients receiving SFX-01 are shown in Figure 3.

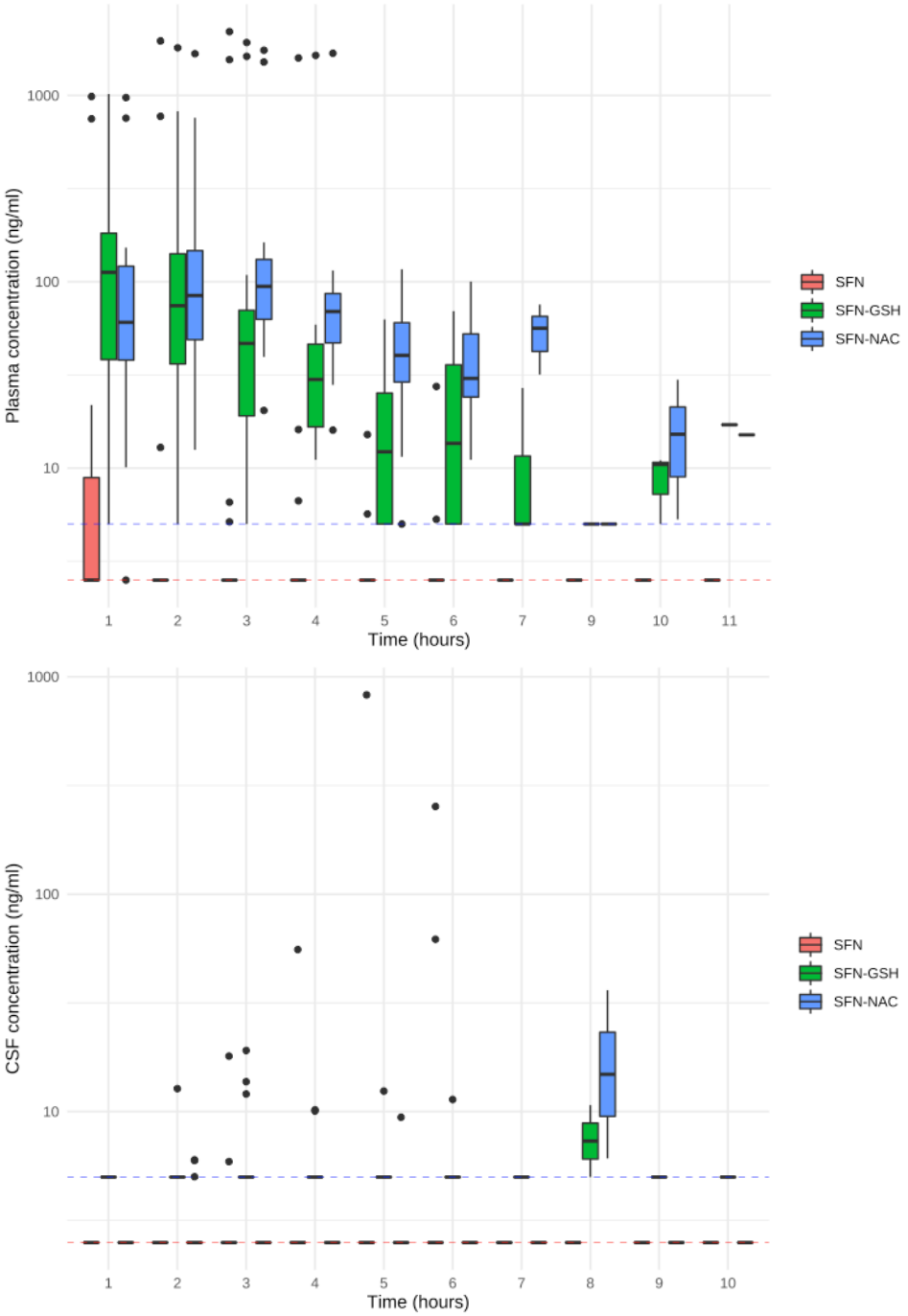


Figure 3. All plasma (a) and CSF (b) SFN, SFN-GSH, SFN-NAC concentrations for all patients in the per protocol population randomised to SFX-01. Values below LLOQ (5ng/ml SFN, 10ng/ml SFN-GSH, 5ng/ml SFN-NAC) are represented as the midpoint between 0 and the LLOQ (dotted red line for SFN and SFN-NAC and blue for SFN-GSH). Median, 25th and 75th percentile and minimum and maximum (minimum or maximum value in the data within 1.5*IQR of 25th or 75th percentile).

Determinants of plasma SFN

Given lower than anticipated CSF SFN and metabolite levels, an exploratory analysis was undertaken to ascertain determinants of plasma and CSF concentrations. First, the total of the plasma SFN and each metabolite (SFN + SFN-GSH + SFN-NAC) was calculated for each patient (using the mean on day 7 for patients with multiple samples in the pharmacokinetic sub-study). The mean total plasma SFN correlated with age (r(41)=0.38, p=0.012, Figure S1), but not weight (r(41)=0.06, p=0.731), height (r(41)=0.09, p=0.580), Body Mass Index (r(41)=0.03, p=0.875, Figure S2) or sex (t(41)=0.52, p=0.606, Figure S3). There was no difference with GSTM1 status (t(40=-0.10, p=0.922), but there was with GSTT1 (t(41)=2.40, p=0.023, Figure 4a&b).

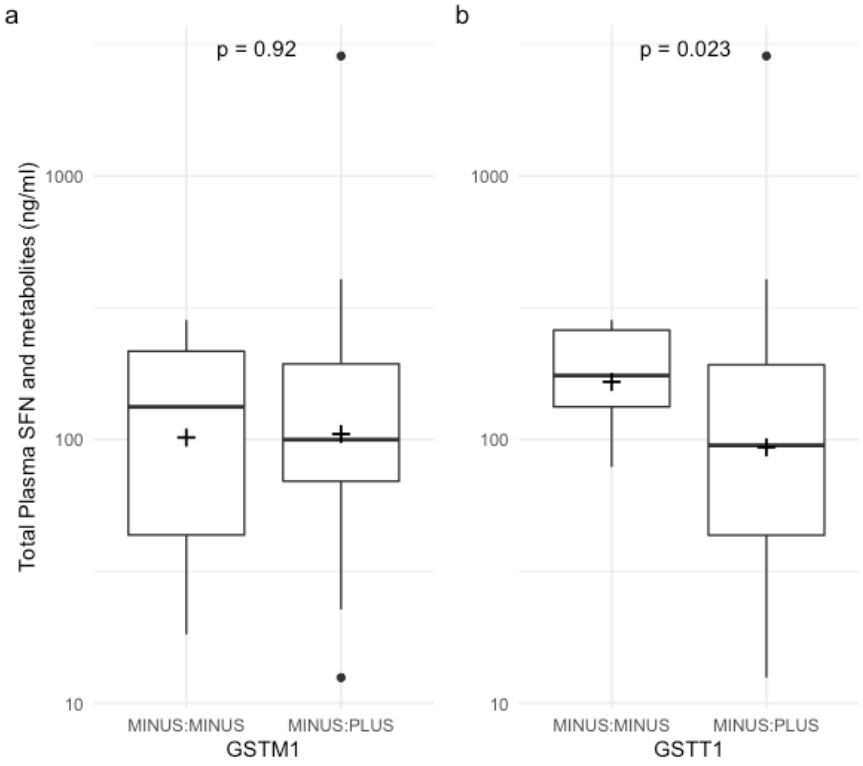


Figure 4. Total plasma SFN and metabolites and a) GSTM1 and b) GSTT1 status. P-value on t-test. Median, 25th and 75th percentile and minimum and maximum (minimum or maximum value in the data within 1.5*IQR of 25th or 75th percentile). Means depicted with +.

Tobit regression showed age and GSTT1 explained 9.1% of variance (R2=0.091, Wald=12.3, p=0.002) in mean total plasma SFN and metabolites. Age (β=0.035, p=0.003) and GSTT1 status (β=-0.666, p=0.039) were independent predictors.

Determinants of CSF SFN

Due to infrequent detection of SFN in CSF, patients were dichotomised as having SFN or metabolites detected in any of their day 7 samples, or not. Backward logistic regression to predict the presence of CSF SFN or metabolites above the LLOQ with total plasma SFN and metabolites, CSF sampling method and QAlb as predictors, showed only QAlb was associated (pseudo R2=0.182, Wald=2.060, p=0.039).

Otherwise, there were no differences between patients with or without detectable CSF SFN or metabolites in age, BMI, WFNS, blood volume on CT, endovascular/microsurgical treatment (Figure S4), CSF sampling method (LP or EVD) (Figure S5), or drop in red blood cells between first and fourth CSF samples (where obtained by LP) (Figure S6).

Haptoglobin and Malondialdehyde

There was no difference in CSF haptoglobin (SFX-01 2.27 vs placebo 1.17 mg/L, ratio 1.981 95%CI 0.992-3.786, p=0.052) or malondialdehyde (SFX-01 0.116 vs placebo 0.103 g/L, ratio 1.123 95%CI 0.747-1.687, p=0.572). Serum, plasma and CSF levels are in Table S3.

Transcranial Doppler Ultrasound

Maximum MCA flow velocity did not differ between groups (ratio of means 1.046 95% CI 0.903-1.211 p=0.545, Table S4). There were no differences on MRMM analysis at each time point (Table S5 and Figure 5).

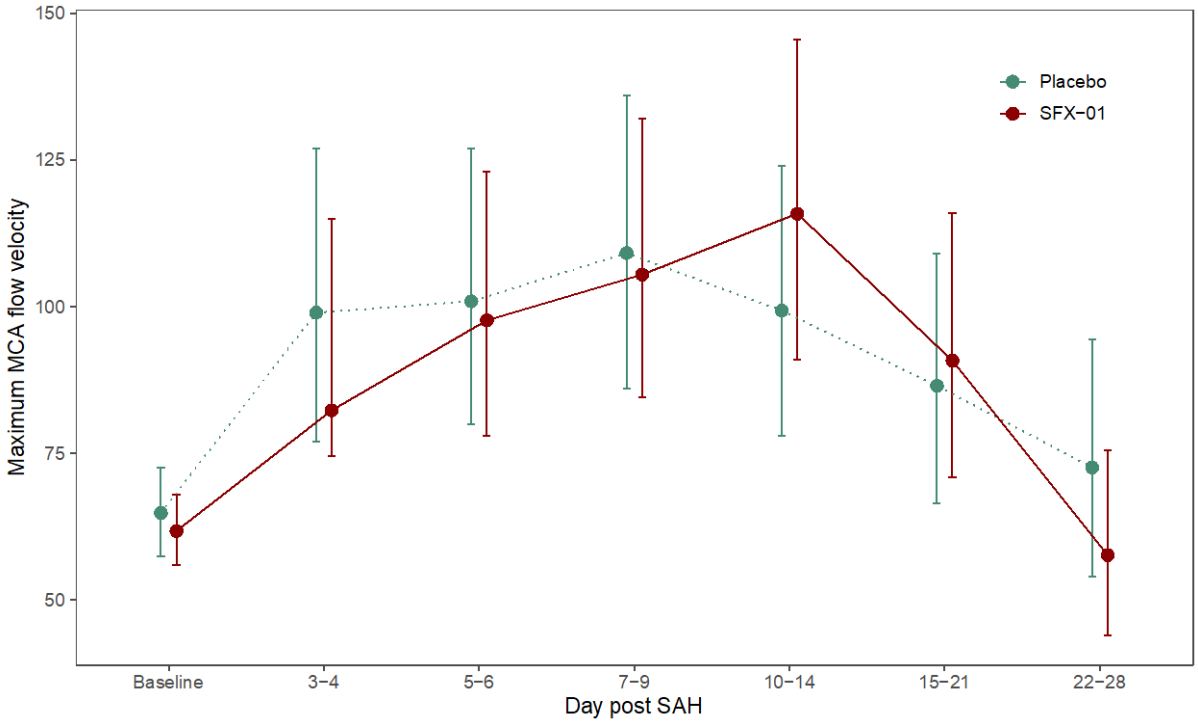


Figure 5. Plot of geometric least squares means over time by treatment for the middle cerebral artery (MCA) mean flow velocity in the per protocol population. Bars indicate the

95% confidence interval. Geometric mean and CI based on standard error of baseline mean is presented at baseline.

Clinical Outcomes

There was no difference in the incidence of DCI, use of hypertensive therapy, mRS (Figure 6), GOSE, SF-36, SAHOT, BICRO-39 or CLCE-24 between groups at any timepoint (Tables S6-S10).

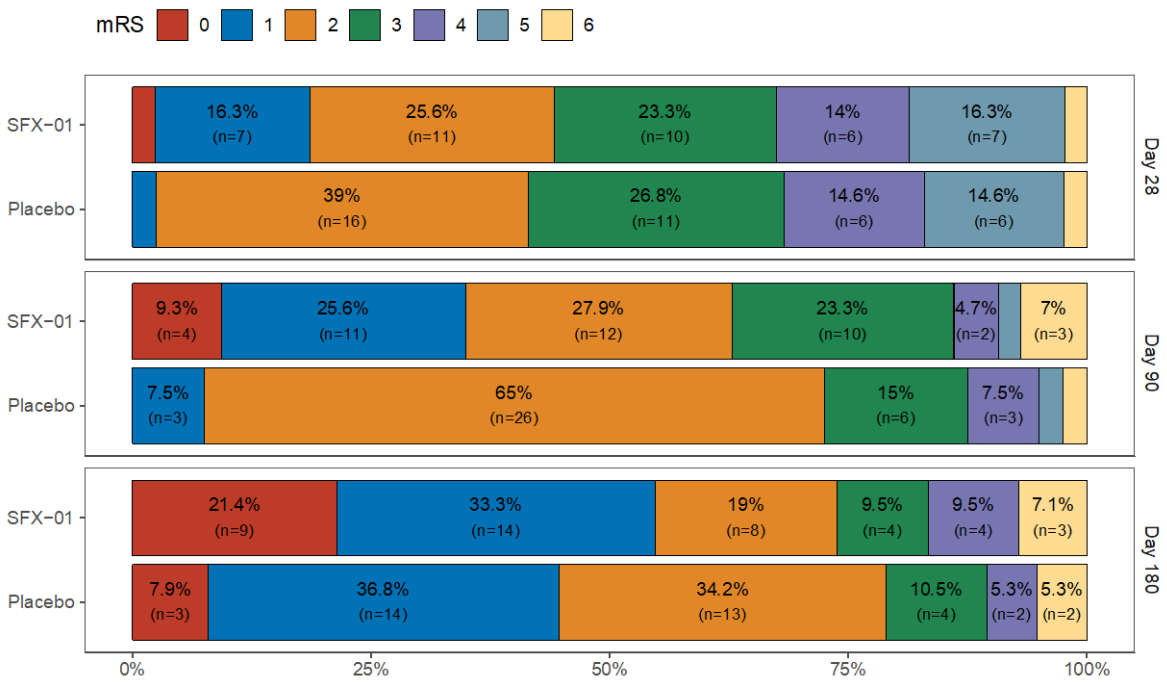


Figure 6. Stacked Bar Chart for Modified Rankin scores at day 28, 90 and 180 in the per protocol population.

Discussion

In this multicentre double blinded RCT, we show oral SFX-01 can be safely administered to an acutely unwell SAH population, with few side effects and excellent plasma concentrations of sulforaphane and its metabolites. CSF penetration was lower than anticipated and we were unable to demonstrate engagement of target mechanisms in the brain (haemoglobin scavenging, oxidative stress, vasospasm) or detect signals of improved clinical outcome.

The safety data is very reassuring, and while SFN and SFX-01 are known to have excellent safety profiles, this was the first time either had been used in large numbers in an acutely unwell population, with many patients intubated in intensive care. This population has high rates of adverse events including haemodynamic, respiratory, haematological, biochemical, and infective. None were worsened by SFX-01.

GI dysmotility and gastric intolerance are common in critically ill patients[27], in SAH and with high dose SFN. It was therefore reassuring that with SFX-01 (often given via nasogastric tube) only a small increase in nausea was seen and that plasma SFN levels were similar to smaller studies in healthy participants[28].

CSF levels were lower than anticipated. Many samples were below LLOQ. For this reason, it was difficult to estimate what typical CSF levels were, and we can only comment that they were at least an order of magnitude lower than plasma levels. This was surprising given the extensive literature on SFN for CNS conditions in animals. Few of these have quantified SFN,

probably because it is a small lipophilic molecule assumed to readily cross the BBB. There is only one animal study of CSF SFN (none in humans). This found SFN in brain homogenate was 5-fold lower than in plasma[21]. There are multiple possible technical explanations for this discrepancy. Animals in that study were not perfused, hence a contribution from intravascular blood would have led to artefactually high estimates of brain SFN concentration. The SFN doses used in the animal study were 2 and 10 times higher than in our study (based on body surface area conversion) and the relationship between plasma and brain may not be linear across concentrations. Mouse and human BBB permeability, and therefore distribution, may also differ. It is also important to note that CSF is produced by the choroid plexus, which is a highly metabolically active tissue. It is possible that SFN and its metabolites were consumed by the choroid plexus leading to different brain and CSF concentrations. A microdialysis study to determine SFN penetration in the tissue through the BBB proper, would therefore be valuable.

Given there was considerable interindividual variability of plasma SFN and low CSF SFN levels we undertook exploratory analyses of predictors of plasma and CSF SFN levels. Plasma SFN was correlated with age as is observed with many drugs[30]. There was no correlation with sex, weight, height, or BMI. Although unusual, some drugs do not show any relationship with size[31]. It means weight-based dosing is unlikely to improve CSF concentrations. SFN is metabolised by GST. Two common gene polymorphisms are GSTM1 and GSTT1 deletions. Their reported relationship to urinary SFN metabolite excretion varies[32,33], and one study of 16 volunteers reported plasma SFN was higher in GSTM1 null individuals[34]. We saw no similar relationship but did observe a more marked increase in GSTT1 null individuals.

The only other explanation of plasma variability that we did not specifically study was the influence of mode of drug delivery (oral or nasogastric tube). While drugs like nimodipine are widely given nasogastrically after SAH, this may influence bioavailability. However, there are considerable confounders in any analysis of this given patients fed by NG tube are much more likely to have gastric dysmotility. Moreover, the majority of patients would have had periods fed orally and periods via nasogastric tube without washout period making comparisons difficult.

We next examined if variability in CSF SFN was related to plasma SFN or QAlb (surrogate for BBB permeability). QAlb did predict the presence of SFN in CSF. This would be in keeping with it having limited BBB permeability. Furthermore, plasma SFN did not predict its presence in the CSF which suggests it is driven more by blood brain barrier permeability than plasma concentration (or alternative mechanisms like active transport out of the central nervous system by BBB transporters as seen with some drugs but not reported for sulforaphane).

BBB permeability can be modelled in silico[29], and models support that permeability to SFN and its metabolites may not be as free as had previously been thought. They predict SFN has borderline permeability (personal communication with Andriy Kovalenko) and that SFN-GSH is permeable and SFN-NAC impermeable. The only other explanations of why CSF levels in this study were low remain either technical which we believe we have ruled out, or due to consumption in the choroid plexus which we are unable to prove or disprove with the available data.

Options for increasing CSF SFN by increasing the oral SFX-01/SFN dose are limited. The dose used was selected based on allometric scaling and is slightly higher than the most common dose in rodent studies in SAH and other CNS conditions. Human phase 1 studies showed increasing gastrointestinal adverse events at higher doses. Increasing frequency of dosing is also unlikely to increase efficacy of SFN, since it acts through transcriptional upregulation. The only remaining option would be to deliver SFX-01 intrathecally but this route is not available to the majority of patients and would require extensive further preclinical testing that is not currently being pursued.

Although CSF SFN levels were low, this does not necessarily mean they were not high enough to engage target pathways. Target engagement has been demonstrated with 5mg/kg SFN in rodents[35], equating to 60% of the SAS dose. This rodent dose is widespread in the literature supporting SFN in neurological conditions, despite no quantification of brain or CSF levels in any of these studies[12,16,17,18,11]. It has specifically been shown to increase haptoglobin (a haemoglobin-binding protein essential for its neutralisation and degradation) by 1.6-fold in the brain[6]. It is therefore intriguing we observed a similar numerical difference of 2-fold in CSF haptoglobin levels (p=0.052). However, we did not see any similar increase in serum levels or reduction in oxidative stress (malondialdehyde), or vasospasm, or any downstream clinical outcomes.

Given these possible signals, it would be interesting to have further assessment of engagement of the Nrf2 pathway and blood samples were obtained for transcriptomic analysis. However, the compartment of interest is the CNS. Transcriptomic analysis here is complicated by the presence of a large number of cells in the blood clot making

interpretation of CSF transcriptomics potentially difficult and requiring brain biopsies which would not be practical. Transcriptional activity in the CNS is therefore best assessed by protein expression. However, most proteins are intracellular and their concentrations likely to be confounded by their release with cellular damage after SAH. Haptoglobin is secreted which made it particularly suited as a readout in addition to the direct relevance of its biological effects to SAH.

Ultimately, it is not possible to confidently conclude whether the lack of clinical benefit in this trial was due to inadequate CSF penetration, SFN's failure to engage target mechanisms, or if-SAH provides such a strong stimulus for Nrf2 activation that the pathway is fully saturated and SFN provides no additional benefit (although this would be contrary to the animal literature[12,16]), or if effect sizes were just too small to detect.

Going forwards, options to better target Nrf-2with sulforaphane in patients with SAH are limited. We chose what appears to have been the optimal/maximal dose for the existing formulation given side effects were just beginning to develop in a small subset of patients. Dosing based on weight or BMI is unlikely to significantly alter this given the lack of association with plasma levels. Given we saw relatively little signal in any of the efficacy endpoints it is unlikely a formulation change will be enough to overcome this. Haptoglobin was the only endpoint showing promise, but any change would need to be orders of magnitude larger to overcome the haemoglobin released by the clot and impact clinical outcome. The only real option would be to consider intrathecal sulforaphane, but this would come with considerable additional complexity.

Limitations

The study was limited to Fisher grade 3 and 4 patients to select those with high blood load and greatest benefit from the intervention and may not be generalisable to all SAH. The Fisher scale also has many limitations. However, we have additionally quantified blood volume on CT, confirming it was a homogenously severe SAH population.

Patients were enrolled up to 48 hours after ictus. While that may seem long, it is shorter than most SAH studies and actual time to treatment was much shorter (29.1 hours). Given that many effects of SAH occur after 72 hours and SFN upregulates the Nrf2 transcriptome within hours, it is unlikely earlier administration would have altered our findings.

Vasospasm as adjudged with transcranial doppler ultrasound was used as a primary outcome. It is increasingly recognised that the contribution of large vessel vasospasm to poor outcome is limited and not all vasospasm leads to poor outcome. An alternative measure such as delayed cerebral ischaemia may have been more clinically relevant. However, given this was a Phase 2 study it was optimised to detect engagement of biological mechanisms (and not clinical outcome). SFX-01 mechanism of action is to improve haemoglobin clearance and reduce oxidative stress. This should be reflected by a reduction contraction in cerebral vessels and reduction in MCA flow velocity irrespective of whether this would prove the mechanism by which it improved outcome. This process is continuous and not binary. Preserving the continuous nature of MCA flow velocity gives it considerably greater statistical power to detect a difference in groups compared to a binary outcome like delayed cerebral ischaemia (or a binarized MCA flow velocity to create a vasospasm vs no vasospasm group) and was therefore much more likely to be able to detect if SFX-01 was

having a biological effect or not and was therefore selected for the purposes of this phase 2 study to reduce sample size.

Despite groups being randomised, there was some imbalance in number of patients with posterior circulation aneurysms between treatment arms. This has occurred by random chance. It could be hypothesised that posterior circulation aneurysms result in a less pronounced increase in MCA flow velocity than those in those in the anterior circulation and this could have altered our findings. However, given there were more patients in the treatment group with posterior circulation aneurysms this would have only increased the chance of seeing a difference between groups - which was not observed. The imbalance could also have led to more patients expected to have poor outcome being randomised to the SFX-01 group and thereby have masked observation of a clinical effect of the treatment, but the differences in outcome between anterior and posterior circulation aneurysms are small and we think this unlikely to have been the case.

15 patients in the intention to treat safety analysis did not meet the criteria for the per protocol analysis. The majority of these were due to patient death before completion of the course of the study drug. The remainder were due to patient withdrawal from the study or missing drug doses. These are inherent in clinical trials necessitating separate analyses. Intention to treat analysis was deemed most appropriate for safety, but per protocol analysis more appropriate for mechanistic understanding.

Sampling from either EVD or LP, rather than one CSF source, is a limitation given the known differences in lumbar and ventricular CSF composition. However, a study limited to patients with EVDs would take longer to complete and be less generalizable. Moreover, analyses

corrected for CSF source. LP could also result in higher lumbar SFN concentrations due to contamination by blood, although we found no relationship with the degree of trauma.

While sample timing in the pharmacokinetic sub-study was carefully controlled, CSF samples in the whole study population were not standardised relative to dosing. This was because their primary purpose was to study downstream mechanisms sensitive to day after SAH (but not time of day), and the anticipated practical difficulties performing LPs in a tight time window. Paired CSF and plasma samples were therefore obtained at random times after SFX-01 dosing and while this represents a random spread, there was a paucity of samples within one or two hours when levels are likely to have peaked.

As described, some analyses were undertaken post-hoc to try to better understand unanticipated findings in the prospectively defined outcomes. These analyses are inherently exploratory in nature.

Finally, while a GLP certified laboratory performed SFN quantification, the assays are limited by their LLOQ. Therefore, although many samples were below LLOQ, this does not mean that SFN or its metabolites were absent, and an assay with a lower LLOQ may have detected the analytes.

Conclusion

SFX-01 is a safe drug for delivery of SFN in acutely unwell patients achieving excellent plasma levels. Plasma levels are influenced by age and GSTT1 status but not weight or BMI.

SFN CSF penetration is lower than expected and was not related to plasma levels but was related to BBB permeability suggesting it cannot be simply overcome by higher dosing or dosing to weight. Other than increasing CSF haptoglobin, there was little evidence SFN engaged target pathways in the CNS with no influence on middle cerebral flow velocity or oxidative stress and no influence on clinical outcome. More research is needed into the CNS penetration of SFN and its metabolites.

Declarations

Acknowledgements

We thank Andriy Kovalenko and Dipankar Roy from the University of Alberta for their in-silico analysis of BBB permeability.

Ethical Approval

The trial was approved by the National Research Ethics Service (Southern Central Hampshire A) and Medicinal Health Care Authority (MHRA), and was conducted in accordance with the Declaration of Helsinki and met the international criteria for Good Clinical Practice. Informed consents were obtained from patients or legal representatives. The trial was registered on clinicaltrials.gov (NCT02614742) and the Consolidated Standards of Reporting Trials (CONSORT) was followed.

Funding

The SAS study was funded by Evgen Pharma

Disclosures

Diederik Bulters and Ian Galea have received support for their research from BPL and CSL Behring

David Howatt and Stephen Franklin were employees of Evgen Pharma

Patrick Garland is employed by Kedrion

Data availability

Requests for derived data supporting the study findings will be considered by the corresponding author.

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CONFIDENTIAL

SFX-01 AFTER SUBARACHNOID HAEMORRHAGE

(SAS STUDY)

PROTOCOL

Version:	Version 7
Version date:	16th May 2018
Superseded version:	Version 6 (22nd December 2017)
Sponsor:	Evgen Pharma plc
Product:	SFX-01
Study Number(s):	EVG001SAH
	EudraCT number: 2014-003284-38

Amendment History

The original protocol was issued 26 November 2015. Amendments are listed beginning with the most recent.

Amendment		Implemented
6	Contact Names and Addresses Medical Monitor Contact details updated Diamond Pharma Services Contact details updated	V7 16th May 2018
6	2.4. Secondary Outcome Endpoints Correction of inconsistency: Removal of SFN analysis for alternate day EVD sampling; addition of Proteomic & Genomic analysis alongside HP & MDA sampling at D7	V7 16th May 2018
6	7.7. Patients with an External Ventricular Drain fitted: Ongoing EVD single sampling (on alternate days +/- 1day from day of EVD fitting until D14 or until the EVD is removed) Correction: deletion of SFN levels from alternate day blood and CSF sampling in EVD group	V7 16th May 2018
6	7.10. Day 28 post ictus (-6/+2 days): Correction: deletion of blood sampling for SFN level	V7 16th May 2018
6	7.15. Schedule of Assessments Clarification that alternate day paired CSF/Blood samples are analysed for HP & MDA/ HP & MDA & Proteomic & Genomic respectively	V7 16th May 2018
6	8.1 Safety measurements Correction: Lipid Profile LDH to LDL	V7 16th May 2018
6	8.3 PK measurements Clarification: Sample schedule	V7 16th May 2018
6	8.6.2 Pregnancy Testing Clarification: menstrual status	V7 16th May 2018
6	8.6.4 EVD Sampling Clarification that the CSF sample taken at day 7 is approximately 20 ml.	V7 16th May 2018
5	Section 4.1 Subject Recruitment, 7.2. Pre-Dose Assessment (within 48 hours of ictus) & 14 INFORMED CONSENT	V6 22nd December 2017

	Clarification that emergency dosing (first two doses) is only permissible where local regulations allow; if local regulations do not allow emergency dosing without consent the patient shall not be enrolled into the study.	
5	Section 3.2 Duration of Treatment, 7.9 Within 2d of discharge, Schedule of Assessment and Synopsis "discharge" defined: Discharge is where the consultant responsible for the intervention of treating the SAH decides that their specialist care is no longer needed and they can safely transfer that care to another health care professional or send the patient home.	V6 22nd December 2017
5	Section 3.2. Duration of Treatment, Section 6.3. Packaging & Storage & 6.6. Drug Storage Clarification that storage conditions are 2-8^{o}C (and that patients will be provided with a cool bag for transportation of the IMP to home)	V6 22nd December 2017
5	Section 3.3.3. Replacements Clarification that patients that have potentially received insufficient or incorrect study medication may be replaced.	V6 22nd December 2017
5	Synopsis & Section 4.2 Inclusion Criteria: Clarification: 1. Patients with radiological evidence of spontaneous **aneurysmal** SAH	V6 22nd December 2017
5	Section 9. ADVERSE EVENT REPORTING Clarification that All AEs will be reported until 30 days after the last dose AEs occurring after 30 days must also be reported if considered related to study drug.	V6 22nd December 2017
5	11.1. Sample Size Addition that up to 120 patients may be recruited and enrolled into the trial in order to provide 90 who will meet the per protocol criteria	V6 22nd December 2017
4	Section 1.7 Population Clarification: The population to be studied are patients with spontaneous aneurysmal SAH	V5 31st October 2017
4	Section 2.4. & 11.2.2 Secondary Outcome Endpoints Addition of Proteomic & Genomic blood sampling alongside HP & MDA at (pre dose 0-48 hours), D7 and D28 (All patients) Addition of paired SFN / SFN metabolite determination in alternate day paired CSF/Blood samples in EVD patients	V5 31st October 2017

	EVD subset: Baseline Serial paired $CSF_{(EVD)}$/blood SFN/SFN metabolite concentration may be taken at one of the first 3 doses	
4	Section 3.1. Overall Study Design and Plan Description Clarification that there were no dispensing errors in the Per Protocol population.	V5 31st October 2017
4	Section 5. CONCOMITANT MEDICATIONS Removal of statement that no reproductive toxicology have been performed with SFX-01	V5 31st October 2017
4	Section 6.3. Packaging & Storage Clarification on delivery of IMP to sites. Update to procedure for randomisation: the next lowest numbered bottle within the specified strata must be selected.	V5 31st October 2017
4	Section 6.4. Labelling Update to labelling such that the strata according to the WFNS grading scale score is included.	V5 31st October 2017
4	Section 6.5.1. Randomization Stratification added to study design: "Patients will be allocated to double-blind medication through a stratified randomisation schedule with the strata defined by site and by baseline severity defined by WFNS score of 1-3 or 4 & 5."	V5 31st October 2017
4	7.2. Pre-Dose Assessment (within 48 hours of ictus), 7.4.Post Dose (12-24 hours after first dose), 7.8. Day 7 post ictus (± 1 day), 7.9. Within 2d of discharge, 7.10. Day 28 post ictus (-6/+2 days): Clarification that Safety Bloods are protocol specific	V5 31st October 2017
4	7.3. Dose (within 48 hours of ictus) Clarification that twice daily dosing should occur approximately 12 hours apart	V5 31st October 2017
4	Section 7.6. Ongoing Assessments & 8.5.3. TCD Recordings: Clarification that standard of care safety bloods are acceptable for ongoing assessments. Clarification that TCD readings (to be performed on alternate days (± 1) post ictus (starting day 3 (± 1) until at least Day 7 (± 1), or discharge whichever is sooner. Any additional TCD readings obtained after this point on clinical grounds will also be recorded).	V5 31st October 2017
4	Section 7.7. Patients with an External Ventricular Drain fitted: Ongoing EVD single sampling (on alternate days +/- 1day from day of EVD fitting until D14 or until the EVD is removed): Addition of blood Proteomic & Genomic analysis Addition Limit of up to Day 14 post ictus for EVD single sampling	V5 31st October 2017

	Addition of SFN analysis of CSF samples	
4	Section 7.8. Day 7 post ictus (± 1 day), 7.10. Day 28 post ictus (-6/+2 days): Blood sample analysis to include HP, MDA *and* Proteomic /Genomic.	V5 31st October 2017
4	Section 8.1. Safety Measurements Clarification of safety blood measurements: Biochemistry (Sodium, Potassium, Urea, Creatinine, Glucose, Calcium, Total bilirubin, Alkaline Phosphatase, Alanine Transaminase, Albumin & C-reactive protein), Haematology (Haemoglobin, White Blood Cell count, Neutrophils (absolute), Lymphocytes (absolute) & Platelets), Lipid Profile (LDH, HDL, Triglycerides & Total Cholesterol), Coagulation Status (PT (or INR) & APTT (or APTR) &Fibrinogen) Clarification that urine microscopy will be performed in accordance with local procedures.	V5 31st October 2017
4	Section 8.3. PK Measurements Addition of Proteomic /Genomic analysis of blood samples.	V5 31st October 2017
4	25. DATA SAFETY MONITORING BOARD (DSMB) Clarification that The DSMB can meet at any point deemed necessary	V5 31st October 2017
4	Synopsis updated to reflect amendments to protocol	V5 31st October 2017
3	Synopsis Typographical correction: Primary Objective, Safety: "To evaluate the safety of up to 28 days of SFX-01 dosed at up to 96 mg Sulforaphane (SFN) per day" amended to "To evaluate the safety of up to 28 days of SFX-01 dosed at up to 92 mg Sulforaphane (SFN) per day"	V4 16th January 2017
3	Synopsis, Schedule of Assessments(footnotes), Section 7.6.Ongoing Assessments Ongoing Assessments: Requirement for timing of assessments amended from "alternate days (±1) post ictus (starting day 3 (±1)) until no longer clinically indicated" for all ongoing assessments to "to be performed on alternate days (± 1) post ictus (starting day 3 (± 1) until at least Day 7 (± 1)) and then until no longer clinically indicated. Any additional TCD readings obtained after this point will also be recorded" for ongoing TCD only	V4 16th January 2017
3	Synopsis & Protocol Section 4.4.1. Replacement of Withdrawn Patients Addition: Replacement of randomised subjects withdrawn prior to completion of day 7 (post ictus) to be discussed (by the Investigator and Sponsor) and approved	V4 16th January 2017

	by the Investigator and Sponsor's Medical Monitor on a case by case basis	
3	Synopsis, Protocol Section 7.2 Pre-Dose Assessment (within 48 hours of ictus), 8.6.1. Blood Sampling, Schedule of Assessments Addition of INR or PT, APTR or APTT, & Fibrinogen (Clauss or Derived) at Pre-Dose Assessment	V4 16th January 2017
3	Synopsis, Protocol Section 2.3 Primary Outcome Endpoints, Section 7 Study Plan, 8.6.1. Blood Sampling & 11.2.1 Statistical parameters and tests /Primary outcome measurements, Schedule of Assessments Coagulation tests amended – PT & APTT updated to INR or PT, APTR or APTT, & Fibrinogen (Clauss or Derived)	V4 16th January 2017
3	Synopsis, Protocol Section 2.3 Primary Outcome Endpoints, Section 7 Study Plan, 11.2.1 Statistical parameters and tests /Primary outcome measurements, Schedule of Assessments Removal of Urine Dipstick test	V4 16th January 2017
3	Synopsis, Protocol Section 2.3 Primary Outcome Endpoints, Section 7 Study Plan, Section 8.1 Safety Measurements, 8.6.1. Blood Sampling & 11.2.1 Statistical parameters and tests /Primary outcome measurements, Schedule of Assessments Removal of lipid tests	V4 16th January 2017
3	Synopsis, Protocol Sections 7.10 Day 28 post ictus & 7.15 Schedule of Assessments Day 28 visit window updated to -6/+2 days	V4 16th January 2017
3	Section 9.8 Adverse Reaction to SFX-01 & 9.9 Serious Adverse Events Addition of email contact details	V4 16th January 2017
2	Clarification that sub-study patients must have EVD fitted prior to randomisation: Synopsis: A sub-study will be conducted in up to 12 patients where an External Ventricular Drain (EVD) fitted. Changed to A sub-study will be conducted in up to 12 patients that already have an External Ventricular Drain (EVD) fitted prior to randomisation; A group of up to 12 patients, all of whom have been fitted with an EVD as part of their normal treatment will be selected for a pharmacokinetic sub-study Changed to	V3 18 Mar 2016

	A group of up to 12 patients, all of whom have been fitted with an EVD as part of their normal treatment and prior to randomisation, will be selected for a pharmacokinetic sub-study Main protocol 7.13: A group of up to 12 patients, all of whom have been fitted with an EVD as part of normal treatment, will be selected for a pharmacokinetic sub-study Changed to A group of up to 12 patients, all of whom have been fitted with an EVD as part of normal treatment prior to randomisation, will be selected for a pharmacokinetic sub-study Main protocol 8.6.4: Twelve patients will participate in a Pharmacokinetic sub-study, all of whom have been fitted with an EVD as part of normal treatment. Changed to Twelve patients will participate in a Pharmacokinetic sub-study, all of whom have been fitted with an EVD as part of normal treatment and prior to randomisation. Synopsis and main protocol 7.13: Deleted: If the EVD is fitted after first dose, serial sampling will only take place at day 7. If the EVD is fitted after the first three doses, serial sampling will only take place at day 7.	
	Synopsis and main protocol 4.3: Addition of exclusion criterion 12 - Known hypersensitivity to any component of a sulforaphane containing product including broccoli	V3 18 Mar 2016
	Synopsis and main protocol 25: Clarification to DSMB requirements for meeting. Deleted: The DSMB must meet as soon as there have been 2 SAEs that are, at least, possibly linked to the administration of SFX-01 The DSMB will consider recommending that the study is placed on hold or stopped if the adverse events associated with participation in the study outweigh the potential benefits of the treatment Changed to The DSMB will consider recommending that the study is placed on hold or stopped if the adverse events associated with participation in the study are considered unacceptable. The DSMB will review blinded study information which will include Changed to	V3 18 Mar 2016

	The DSMB will review unblinded study information which will include Main protocol: Deleted: The DSMB will also be able to request unblinding of patients. The operating procedure will document the planned flow of information in order to describe how the integrity of the study with respect to preventing dissemination of unblinded study information is assured.	
	Remove reference to CTC AE grading throughout and replace with applicable non-CTC grading scheme: Synopsis and main protocol 2.3: Change in Common Toxicity Criteria (CTC) Changed to Change in grading of AE severity Main protocol 8.1 & 11.2: Escalation in Common Toxicity Criteria (CTC) Change to Escalation in grading of AE severity Synopsis and main protocol 25: The DSMB must meet if 2 patients escalate to grade 4 on the Common Toxicity Criteria scale Changed to The DSMB must meet if 2 patients have a grading change in AE severity (from mild/ moderate to severe or life threatening).	V3 18 Mar 2016
	Procedural clarifications	V3 18 Mar 2016
	Administrative changes	V3 18 Mar 2016
1	Protocol Point 11 of exclusion criteria (synopsis and main protocol) (MHRA request) & Main Protocol section 7.9 Within 2d of discharge Timeframes for use of contraception – 90 days for men and 30 days for women	V2 27th Jan 2016
	Main Protocol section 1.5 Dose Rationale (MHRA request) "In the pre-clinical studies conducted to date" Changed to "In the clinical studies conducted to date"	V2 27th Jan 2016
	Main Protocol section 7.9 Within 2d of discharge (MHRA request) Specifics of what forms of contraception are acceptable	V2 27th Jan 2016
	Synopsis : Pre-Dose Assessment (within 48 hours of ictus) & Main protocol section 7.2 - Clarify procedure: "CT/MRI & Fisher grading"	V2 27th Jan 2016

	Changed to “recording results of CT/MRI & Fisher grading”	
	Synopsis: Between Pre-dose Assessment & Post Dose & Main Protocol section 7.5 - Clarify procedure: “Angiographic assessment (CTA/DSA/MRA) ” Changed to “Recording of results and form of angiographic assessment (CTA/DSA/MRA) “	V2 27th Jan 2016
	Main protocol 7.15 Schedule of Assessments - Typographical Error X added against Safety Urine testing at Pre-dose assessment. (Previously in text but missing from Schedule of Assessments)	V2 27th Jan 2016

Protocol Approval Page

This protocol has been read and approved by:

Sponsor representative:	
Signature:	
Date:	18/5/18

Chief Investigator:	DIEDERIK BUTERS
Signature:	
Date:	17/5/18

The Clinical Trial Company:	J. BOOTH.
Signature:	
Date:	17/5/18

Statistician:	
Signature:	
Date:	18 may 2018

INVESTIGATOR PROTOCOL APPROVAL PAGE

SFX-01 AFTER SUBARACHNOID HAEMORRHAGE

(SAS STUDY)

I, the undersigned, have read and understood the protocol and am aware of my responsibilities as an investigator. I agree to conduct the study in accordance with this protocol and any subsequent amendments, the Declaration of Helsinki, ICH GCP guidelines, and the laws and regulations of the country in which the study is being conducted.

Investigator Name: **Investigator Title:** **Investigator Address:**	

Investigator Signature: **Date:**	

Protocol Synopsis

TITLE:

SFX-01 After Subarachnoid Haemorrhage (SAS)

PROTOCOL NO:

EVG001SAH

STUDY PHASE:

Phase II

INVESTIGATOR STUDY SITES:

This study will be conducted at multiple study sites located in the United Kingdom

OBJECTIVES:

Primary Objectives:

Safety
To evaluate the safety of up to 28 days of SFX-01 dosed at up to 92 mg Sulforaphane (SFN) per day.

Pharmacokinetic
To detect the presence of SFN in Cerebrospinal Fluid (CSF)

Efficacy
To determine if a minimum of 7 days treatment with SFX-01 reduces Middle Cerebral Artery (MCA) peak flow velocity following Subarachnoid Haemorrhage (SAH).

Secondary Objectives:

- To determine if a minimum of 7 days treatment with SFX-01 improves clinical outcome following SAH as measured using the modified Rankin Scale assessed at 7 days, discharge, 28, 90 and 180 days post ictus.
- To determine blood SFN levels (and its metabolites) with treatment with SFX-01 (300mg bid).
- To determine CSF SFN levels and kinetics with treatment with SFX-01 (300mg bid).
- To determine if up to 28 days treatment with SFX-01 increases blood haptoglobin (HP) levels and decreases malondialdehyde (MDA) levels following SAH.
- To determine if up to 28 days treatment with SFX-01 can reduce the incidence of Delayed Cerebral Ischaemia (DCI) following SAH.
- To determine if up to 28 days treatment with SFX-01 improves long-term outcome in subjects following SAH.
- To determine if up to 28 days of treatment with SFX-01 can reduce iron deposition and cortical atrophy following SAH.

METHODOLOGY:

This is a Safety, Tolerability, Pharmacokinetic and Pharmacodynamic Study of SFX-01 in Subarachnoid Haemorrhage, with exploratory evaluations of efficacy.

The study is a randomised, double-blind, parallel-group design comparing SFX-01 (300 mg) taken orally as capsules or as a suspension via a nasogastric tube (NG) twice-daily for up to 28 days versus placebo in up to 120 patients to achieve 90 patients in the per-protocol analysis set, who have had SAH and present within 48 hours of ictus.

The treatment group will receive SFX-01 in order to improve outcome and reduce the long-term complications of SAH such as Delayed Cerebral Ischaemia, as reflected by Trans-Cranial Doppler (TCD) readings. The objective is to demonstrate safety and search for signals of efficacy in patients that have had SAH.

A sub-study will be conducted in up to 12 patients that already have an External Ventricular Drain (EVD) fitted prior to randomisation; serial CSF samples will be taken pre- & post-dose on two occasions to determine pharmacokinetics of Sulforaphane in CSF in comparison with plasma pharmacokinetics. Sub-study patients will undergo all other procedures (with the exception of lumbar puncture).

Treatment duration is up to 28 days; follow up duration is 28 days, three and six months. The planned trial period is 24 months.

The Per Protocol Population (for Primary analysis) will be considered to be those patients that have been dosed for a minimum of 7 days.

A data safety monitoring board (DSMB) will be set-up to monitor safety throughout the trial period and provide recommendations for any necessary actions. A steering committee (comprising the Chief Investigator and the sponsor's Chief Medical Officer) will receive and review the reports from the DSMB, and take action as appropriate.

NUMBER OF PATIENTS:

Up to 120 patients may be recruited and enrolled into the trial in order to provide 90 who will meet the per protocol criteria and be analysed for the efficacy analyses.

In the instance where patients have been entered into the trial prior to informed consent being obtained (i.e. through the emergency consent procedure) and consent is subsequently refused or not obtained within 24 hours by the patient and/or legal representative the participants shall be withdrawn and replaced.

Replacement of patients who withdraw/are withdrawn prior to completion of day 7 (post ictus) is to be discussed (by the Investigator and Sponsor) and approved by the Investigator and Sponsor's Medical Monitor on a case by case basis.

Patients who withdraw for any other reason after randomisation will not be replaced.

INCLUSION/EXCLUSION CRITERIA:

Inclusion criteria

1. Patients with radiological evidence of spontaneous aneurysmal SAH
2. Fisher grade 3 or 4 on CT
3. Definitive treatment of aneurysm has not been ruled out
4. Previously living independently

5. In the opinion of the investigator, the delay from ictus to randomisation and initiation of trial medication will not exceed 48 hours
6. Aged 18 to 80 years
7. In the opinion of the investigator it will be possible to obtain Informed Consent from the Patient, Personal Legal Representative or Professional Legal representative within 24 hours of first dose

Exclusion criteria

1 Traumatic SAH
2 Fisher grade 1 or 2
3 SAH diagnosed on lumbar puncture with no evidence of blood on CT
4 Decision not to treat aneurysm has been made
5 Plan to withdraw treatment
6 Significant kidney disease as defined as plasma creatinine ≥2.5mg/dL (221 μmol/l)
7 Liver disease as defined as total bilirubin ≥2-fold the upper limit of normal; as measured by the local laboratory
8 Females who are pregnant or lactating.
9 Participants enrolled in another interventional research trial in the last 30 days
10 Patients for whom it is known, at the time of screening, that clinical follow-up will not be feasible
11 Patients unwilling to use two forms of contraception (one of which being a barrier method see section 7.9) 90 days for men and 30 days for women after last IMP dose
12 Known hypersensitivity to any component of a sulforaphane containing product including broccoli

DOSE/ROUTE/REGIMEN:

Active: SFX-01 (active 300 mg capsule taken orally twice-daily for up to 28 days)

Placebo: SFX-01 placebo (placebo 300 mg capsule taken orally twice-daily for up to 28 days)

For patients unable to take tablets orally but have a nasogastric tube in situ, the study drug will be administered per tube.

Patients will be randomised to double blind active or placebo Investigational Medicinal Product and will be stratified using the most recent WFNS grading score, post ictus and prior to randomization. The two strata will comprise WFNS scores 1-3 and WFNS scores 4-5.

REFERENCE TREATMENT:

Placebo capsules identical in appearance to SFX-01 active capsules.

CRITERIA FOR EVALUATION:

Primary Outcome Variables

Safety

- Concomitant medication
- Adverse events
- Change in grading of AE severity
- FBC, U&Es, LFT, CRP & Urine Microscopy

- INR or PT, APTR or APTT, & Fibrinogen (Clauss or Derived) at 7 & 28 days

Pharmacokinetic

- Presence of SFN in CSF

Efficacy

- The maximum MCA flow velocity determined using TCD.
  Treatment groups will be compared using a t-test.

Secondary Outcome Variables

- Incidence of Delayed Cerebral Ischaemia (DCI) defined as a new focal deficit or reduction in Glasgow Coma Scale (GCS) ≥2 if not explained by other causes (i.e re-bleed, hydrocephalus, seizure, meningitis, sepsis or hyponatraemia)
  Treatment groups will be compared using a chi-square test.
- Incidence of new cerebral infarct on Computed Tomography (CT) or Magnetic Resonance Imaging (MRI)
  Treatment groups will be compared using a chi-square test.
- Institution of hypertensive (triple H) therapy for presumed DCI
  Treatment groups will be compared using a chi-square test.
- Modified Rankin Scale (mRS), at 7 days, discharge, 28, 90 and 180 days.
  Treatment groups will be compared using a van Elteren test.
- SF-36 quality of life survey at 28, 90 & 180 days.
  Treatment groups will be compared using a t-test.
- Checklist for Cognitive and Emotional Consequences (CLCE-24), Brain Injury Community Rehabilitation Outcomes Scale (BICRO-39), 90 & 180 days.
  Treatment groups will be compared using a van Elteren test.
- Subarachnoid Haemorrhage Outcome Tool (SAHOT) and Glasgow Outcome Scale – Extended (GOSE) at 28, 90 & 180 days.
  Treatment groups will be compared using a van Elteren test.
- Length of acute hospital stay
  Treatment groups will be compared using a Wilcoxon-Mann-Whitney-test.
- Discharge location (e.g. home, rehabilitation centre etc.)
  Treatment groups will be compared using a chi-square test.
- Amount of iron identified on MRI Susceptibility Weighted Imaging (SWI) 180 days after start of treatment.
  Treatment groups will be compared using a t-test.
- Cortical atrophy on T1 MRI at 180 days after start of treatment
  Voxel-based morphometry will be used to identify and quantify regional areas of atrophy

Non-EVD Patients: i.e. patients will have a Lumbar Puncture for collection of CSF

- Blood HP and MDA, Proteomic & Genomic concentration at baseline (pre dose 0-48 hours), D7 and D28.
- Paired $CSF_{(Lumbar\ Puncture)}$/blood HP, MDA, Proteomic & Genomic & SFN/SFN metabolite concentrations at Day 7

EVD Patients: (i.e. will not have a lumbar puncture)

- Blood HP and MDA, Proteomic & Genomic concentration at baseline (pre dose 0-48 hours), D7 and D28.

- Paired $CSF_{(EVD)}$/blood HP & MDA, Proteomic & Genomic concentration on alternate days (+/- 1 day) starting on day of EVD fitting until D14 or the EVD removal.
- Paired $CSF_{(EVD)}$/blood HP, MDA, Proteomic & Genomic & SFN/SFN metabolite concentration at day 7

Subset of 12 EVD Patients: In addition to all other sampling the following samples will be taken:

- Serial paired $CSF_{(EVD)}$/blood SFN/SFN metabolite concentrations at one of the first 3 doses and day 7

Measured PK-variables will be log-transformed, if necessary, and descriptively displayed using Box-plots.

STATISTICAL METHODS:

Primary analysis will be carried out using data from the Per-Protocol Population, i.e. those patients that have been dosed to day 7 post ictus.

Exact definition of major protocol deviations will be discussed by the clinical team case by case during the blind review of the data and described in the blind review document.

Protocol violations will be considered for each protocol period separately.

All data will be presented in patient data listings. For continuous variables, descriptive statistics (n, mean, median, standard deviation, minimum, and maximum) will be presented. For categorical variables, frequencies and percentages will be presented. Graphical displays will be presented as appropriate.

Power and Sample Size:

Ninety patients will give 80% power to detect a difference in maximum MCA flow velocity which is approximately half of the standard deviation of the mean value. The standard deviation is assumed to be approximately 30% of the mean value based on historical data.

Efficacy:

The primary efficacy variable will be compared between the treatment groups using a t-test for maximum MCA flow velocity.

Categorical secondary endpoints will be compared using chi-square-tests.

Pharmacokinetics:

Measured PK-variables will be log-transformed, if necessary, and descriptively displayed using Box-plots.

STUDY SCHEDULE:

Continuous Assessment: Adverse Events, Concomitant Medication & Medication Compliance

Pre-Dose Assessment (within 48 hours of ictus): Informed Consent (within 24 hours of first dose), Inclusion/Exclusion (screen failures to be recorded), recording results of CT/MRI & Fisher grading, Demographics/Medical History, Physical Examination, Pregnancy test, Concomitant Medication, protocol specific Safety Bloods (Biochemistry, Haematology, Lipid Profile & Coagulation Status) and Urine, blood HP, MDA,

Proteomic/Genomic sampling and recording of World Federation of Neurological Societies (WFNS) scale after resuscitation (post ictus and prior to randomisation). WFNS score will be used to allocate stratified randomised medication.

Dose (within 48 hours of ictus): Randomisation & administration of the first dosage (within 48 hours of ictus, twice daily for 28 days)

Post Dose (12-24 hours after first dose): protocol specific Safety Bloods

Between Pre-dose Assessment & Post Dose: Recording of results and form of angiographic assessment (CTA/DSA/MRA) and first TCD reading (to measure peak MCA flow)

Ongoing Assessments – All patients: Safety Bloods as part of normal clinical care, TCD readings (to be performed on alternate days (± 1) post ictus (starting day 3 (± 1) until at least Day 7 (± 1), or discharge whichever is sooner. Any additional TCD readings obtained after this point will also be recorded) and Glasgow Coma Score

Patients with an EVD fitted: Ongoing EVD single sampling (on alternate days (+/- 1 day) from day of EVD fitting until the EVD is removed or up to 14 days): A single EVD sample is to be taken, paired with a single blood sample for determination of CSF/blood HP, MDA, Proteomic & Genomic levels

Day 7 post ictus (± 1 day): Protocol specific Safety Bloods and Urine, TCD reading, modified Rankin Score and Concomitant Medication.

- Non-EVD patients only: Paired blood sampling and lumbar puncture CSF sample to determine CSF/blood HP, MDA, Proteomic/Genomic & SFN/SFN metabolite concentrations
- EVD patients only: Paired blood and EVD CSF sampling to determine CSF/blood HP, MDA, Proteomic/Genomic & SFN/SFN metabolite concentrations

Discharge (-2 days): Protocol specific Safety Bloods and Urine, modified Rankin Score, Glasgow Coma Score and Concomitant Medication.
Note: Discharge is defined as when the consultant for the intervention of treating the SAH decides that their specialist care is no longer needed.

Day 28 post ictus (-6/+2 days): Modified Rankin Score, Short Form 36 Health Survey, Subarachnoid Haemorrhage Outcome Tool, Glasgow Outcome Scale (Extended) and Concomitant Medication. Protocol specific Safety Bloods and Urine, HP, MDA, Proteomic/Genomic.

Day 90 post ictus (± 14): Modified Rankin Score, Short Form 36 Health Survey, Checklist for Cognitive and Emotional Consequences, Brain Injury Community Rehabilitation Outcomes Scale, Subarachnoid Haemorrhage Outcome Tool, Glasgow Outcome Scale (Extended) and Concomitant Medication.

Day 180 post ictus (± 28): Modified Rankin Score, Short Form 36 Health Survey, Checklist for Cognitive and Emotional Consequences, Brain Injury Community Rehabilitation Outcomes Scale, Subarachnoid Haemorrhage Outcome Tool, Glasgow Outcome Scale (Extended) and Concomitant Medication. An MRI will be performed within 60 days of the Day 180 visit

Pharmacokinetic sub-study
A group of up to 12 patients, all of whom have been fitted with an EVD as part of their normal treatment and prior to randomisation, will be selected for a pharmacokinetic sub-study.

Additional patient consent will be required for the sub-study; in cases where patients lack capacity the consent of a Personal Legal Representative will be sought before any sub-study procedures are carried out.

The patients will, in addition to all other protocol required procedures of a patient with an EVD fitted, undergo serial paired blood/CSF sampling (1 sample pre dose and hourly ± 5 minutes for 6 hours post dose) after one of the first three doses and on day 7 after the morning dose.

SAFETY

Recruitment:
The DSMB will convene after 20 patients have been dosed to day 7 post ictus (with adequate safety assessment data) as in-patients in tertiary care for a formal safety review.

The safety review shall make a decision on the acceptability of discharging patients from tertiary care with SFX-01 to complete the dosing course to day 28.

Data Safety Monitoring Board

The DSMB will convene under the following circumstances:

- The DSMB must meet once the 20th patient has been dosed to day 7 post ictus
- The DSMB must meet as soon as there has been a SUSAR
- The DSMB must meet if 2 patients have a grading change in AE severity (from mild/ moderate to severe or life threatening).

Study Stopping Rules

The clinical investigation can be placed on hold / stopped early for two reasons and will be based on clinical judgement:

- The DSMB will consider recommending that the study is placed on hold or stopped if the adverse events associated with participation in the study are considered unacceptable.
- The DSMB will consider recommending that the study is placed on hold or stopped if the adverse events associated with SFX-01, in their opinion, significantly outnumber (in frequency or intensity) the adverse events associated with the normal standard of care.
- At any point deemed necessary

Contact Names and Addresses

Sponsor:	Evgen Pharma plc
Registered Address:	Liverpool Science Park IC2 146 Brownlow Hill Liverpool L3 5RF
Contact details:	Tel: +44(0) 151 705 3532 Fax: +44(0) 151 705 3534
Head Office	The Colony Altrincham Road Wilmslow Cheshire SK9 4LY
Contact details:	+44 (0) 1625 466591
Personnel:	Dr Thomas Morris - Medical Monitor
Chief Investigator	Mr. Diederik Bulters MBChB and intercalated BSc (pharm), Consultant Neurosurgeon and Honorary Senior Clinical Lecturer
Address:	University Hospital Southampton, Tremona Road, Southampton, Hampshire, SO16 6YD
	Tel: +44(0) 2381 205 311
	Fax: +44(0) 2381 204148
CRO:	The Clinical Trial Company Ltd
Address:	Mere View Barn, Park Lane, Pickmere, Knutsford, Cheshire, WA16 OLG
Contact details:	Tel: +44(0) 1565 733 772
	Fax: +44(0) 1565 732 958
Personnel:	Ewan Campbell – Director
	Ingrid Gerber – Project Manager
Statistician	Data Magik Limited
Address:	Laburnum House, East Grimstead, Salisbury, Wiltshire, SP5 3RT
Personnel:	David Fleet
Telephone	+44 (0)1722 712 972
Pharmacovigilance	Diamond Pharma Services Ltd
Address:	Suite 2, Ground Floor, Field House, Station Approach, Harlow, Essex, CM20 2FB
Telephone	+44 (0) 203 911 9410
Fax	+44 (0)1279 418964

Abbreviations

AE	Adverse Event
ADL	Activities of Daily Living
APTR	Activated Partial Thromboplastin Time Ratio
APTT	Activated Partial Thromboplastin Time
AUC	Area Under the Curve
bid	Two times daily
BICRO	Brain Injury Community Rehabilitation Outcomes Scale
CFR	Code of Federal Regulations
CI	Chief Investigator
CLCE	Checklist for Cognitive and Emotional Consequences (CLCE-24)
eCRF	Electronic Case Report Form
CRP	C-Reactive Protein
CSF	Cerebrospinal Fluid
CT	Computed Tomography
CTA	CT Angiography
DCI	Delayed Cerebral Ischaemia
DSA	Digital Subtraction Angiography
DSMB	Data Safety Monitoring Board
EVD	External Ventricular Drain
FBC	Full Blood Count
FDA	Food and Drug Administration (United States)
GCP	Good Clinical Practice
GCS	Glasgow Coma Scale
GI	Gastro-Intestinal
GOSE	Glasgow Outcome Scale (Extended)
GP	General Practitioner
HO-1	Haeme Oxygenase-1
HP	Haptoglobin
HPMC	Hydroxypropyl Methycellulose
IB	Investigator Brochure
ICH	International Conference on Harmonisation
IMP	Investigational Medicinal Product
INR	International Normalised Ratio
LFT	Liver Function Test(s)
MCA	Middle Cerebral Artery
MDA	Malondialdehyde
MRA	Magnetic Resonance Angiography
MRI	Magnetic Resonance Imaging
mRS	Modified Rankin Scale

Nrf2	Nuclear factor erythroid 2-related factor 2
NG	NasoGastric
NICU	NeuroIntensive Care Unit
NOAEL	No-observed-adverse-effect level
NQO1	NAD(P)H:quinone oxidoreductase-1
PK	Pharmacokinetic
PT	Prothrombin Time
REC	Research Ethics Committee
SAE	Serious Adverse Event
SAH	Subarachnoid Haemorrhage
SAHOT	Subarachnoid Haemorrhage Outcome Tool
SF-36	Short Form (36) Health Survey
SFN	Sulforaphane
SFX-01	The Investigational Medicinal Product/stabilised Sulforaphane
SUSAR	Suspected Unexpected Serious Adverse Reaction
SWI	Susceptibility Weighted Imaging
TCD	Trans-Cranial Doppler
TCTC	The Clinical Trial Company Ltd
TMF	Trial Master File
TSF	Trial Site File
U&Es	Urea & Electrolytes
WFNS	World Federation of Neurosurgical Societies Scale

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1. BACKGROUND INFORMATION AND STUDY RATIONALE

Spontaneous Subarachnoid Haemorrhage (SAH) is a complex cerebrovascular disease with an incidence of 8-10 per 100,000 population affecting more than 7000 patients within the UK annually. Around 85% of cases are due to ruptured intracranial aneurysms. Perimesencephalic non-aneurysmal SAH accounts for 10% of spontaneous SAH [1]. The incidence of SAH is age-related with higher incidence amongst age group 40-60 years and a peak incidence at the age of 55. SAH carries a high overall mortality rate of up to 67% [2] and only half of the survivors are able to live independently. Given the age-related incidence and high morbidity and mortality, SAH has a high burden on society due to the loss of productivity and resources used [3].

Conventionally following SAH, treatment is primarily directed to securing the aneurysm to prevent further re-bleed. This however does nothing to ameliorate the morbidity and mortality due to the haemorrhage. The only approved effective medicine to reduce morbidity is nimodipine [4]. However, its effects are small and despite its use poor outcomes remain a significant problem as evidenced by contemporary outcome data since its introduction [5]. Moreover, even in survivors conventionally considered to have made a good recovery, neurocognitive deficits are common leading to extensive problems with social reintegration and functioning in the workplace [6].

The mechanisms underlying poor outcomes are multifactorial. A significant component is due to secondary injury from inflammation [7], spreading depolarisation [8], macroscopic cerebral vasospasm and microcirculatory disturbance [9]. The common factor in all these mechanisms is that they are initiated by extracellular haemoglobin released as red blood cells in the clot lyse. This results in direct neurotoxicity and increased oxidative stress and further injury [10]. Thus any treatment to ameliorate their effects would be best targeted at reducing the cell free haemoglobin, oxidative stress and inflammation.

Sulforaphane (SFN) is known to up-regulate the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway; Nrf2 is a promoter of haptoglobin (HP) expression and a wide range of anti-oxidant and anti-inflammatory enzymes including Haeme Oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase-1 (NQO1). These effects have been shown to reduce inflammation and neurological deficits seen in rats after intracerebral haemorrhage and SAH [11, 12].

As SFN is an unstable molecule it cannot practically be employed in clinical use. However when complexed with cyclodextrin to form SFX-01 it is stable and can be practically used in the clinical setting. On ingestion, SFN is released from the cyclodextrin and thus SFX-01 is an effective method to deliver SFN.

1.1. Investigational Agent

SFX-01 (300 mg) taken orally or via nasogastric tube twice-daily

1.2. Preclinical Data

There is limited clinical experience with SFX-01 with two Phase I trials completed to date (EVG001/N & EVG002/N). However, there are numerous trials that have used makeshift SFN preparations derived from broccoli extracts.

1.2.1. Nrf2 pathway

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox sensitive transcription factor known as a protector for many organs, including the brain. Nrf2 is sequestered in the cytoplasm by kelch-like ECH-associated protein 1 (Keap1) under physiological conditions. It transactivates the expression of a group of anti-oxidant and cytoprotective enzymes, such as HO-1, NQO1 and glutathione S-transferase-a1 [13]. In response to oxidative stress it translocates into the nucleus and binds to the antioxidant-response element. This coupling promotes transcription of protective genes encoding antioxidant and detoxifying enzymes [14]. The full range Nrf2-regulated genes has been documented by induction with sulforaphane [15].

Indeed Nrf2 seems a global neuroprotectant; it has been demonstrated to have a key role in intracerebral haemorrhage and cerebral ischaemia, both of which pathophysiologically overlap with SAH.

A previous study utilising a mouse intracerebral haemorrhage model showed that Nrf2 deficient mice were significantly more prone to haemorrhagic brain injury and neurologic deficits. Nrf2 reduces intracerebral haemorrhage induced early brain injury, possibly by providing protection against leukocyte-mediated free radical oxidative damage [12].

Previous studies have shown that Nrf2 upregulation also provides protection from cerebral ischemia *in vivo* [16, 17]. While clinically it is difficult to capitalise on this as it requires pre conditioning at a time when a stroke may not be expected. In SAH, however, ischaemia has a delayed onset and thus there is an opportunity for augmenting Nrf2 expression prior to DCI.

Critically Nrf2 is a key regulator in reducing oxidative stress, inflammatory damage and accumulation of toxic metabolites involved as part of the underlying process in SAH. This has initially been investigated *in vitro* in [11], and more latterly *in vivo* in mice [18, 19]. In this study the absence of Nrf2 function resulted in exacerbated brain injury with increased brain oedema, blood–brain barrier disruption, neural apoptosis, and severe neurological deficits at 24 hr after SAH. Cerebral vasospasm was severe at 24 hr after SAH, but not significantly different between wild type and Nrf2 knock-out mice after SAH. Meanwhile, Malondialdehyde (MDA), TNF-α and IL-1β were increased and GSH/GSSG ratio was decreased in Nrf2 KO mice after SAH.

1.2.2. Sulforaphane (SFN)

Sulforaphane (SFN) is a well-studied isothiocyanate and potent inducer of Nrf2 signalling. It is formed on ingestion of cruciferous vegetables, particularly broccoli and broccoli sprouts. These contain glucoraphanin which is hydrolysed to sulforaphane by myrosinase, (present in the plant as well as the gut microflora). Sulforaphane is known to cross the blood brain barrier in animal models [20]. Experience in humans with sulforaphane from broccoli and broccoli sprouts is extensive with limited safety data. In a placebo-controlled, double-blind, randomized clinical trial of glucosinolates (principally glucoraphanin) as well as isothiocyanates (principally sulforaphane) no significant or consistent subjective or objective abnormal events [21] were reported. Most of the experience has been at lower doses than in the current proposed study.

Sulforaphane has been tested in animal models of ischaemic stroke, intracerebral haemorrhage and subarachnoid haemorrhage. In a rat ischaemic stroke model, SFN was shown to reduce infarct volume following temporary occlusion of left common carotid artery or middle cerebral artery. Those in the treatment group were injected with intraperitoneal SFN 15 minutes after the onset of ischaemia. SFN was found to increase brain Haeme Oxygenase-1 (HO-1) mRNA levels, an enzyme involved in reducing oxidative stress. Oxidative stress is thought to be a

contributing factor in the pathogenesis of ischaemic stroke and inflammation. The overall infarct volume was significantly reduced in the SFN-treated group by as much as 30% [22]. This is particularly relevant to SAH where in addition to territorial ischaemia from large vessel vasospasm, there is increasing recognition of the effect of microcirculatory disturbances and infarction. This was first demonstrated in a SAH post mortem study which showed small cortical and hypothalamic infarcts in most subjects [23]. These insults are too small to discriminate by computed tomography (CT) and thus under-recognised in clinical practice.

Disruption of the blood-brain barrier and cerebral oedema are the major pathogenic mechanisms leading to neurological dysfunction and death after ischaemic stroke. In a study [24] where rats were preconditioned with SFN prior to ischaemic stroke (similar to the model described above) Nrf2 and HO-1 protein expression was shown to be upregulated in cerebral microvessels of peri-infarct regions as well as cerebral endothelium in the infarct core. In animals pre-treated with SFN there was marked reduction of lesion progression, blood-brain barrier disruption and neurological dysfunction. Delayed Cerebral Ischaemia (DCI) as a complication of SAH presents a few days after the onset of symptoms (between days 3-14). Therefore, preconditioning with SFN is a reasonable and feasible method of preventing this phenomenon in those with SAH even if it is not prior to conventional ischaemic stroke.

Oxidative and cytotoxic damage play an important role in the pathogenesis of intracerebral haemorrhage. In one study rats and Nrf2-deficient or control mice received intrastriatal injection of autologous blood to mimic intracerebral haemorrhage. The treatment group was injected with intraperitoneal SFN; activation of Nrf2 with SFN resulted in upregulation of haptoglobin (central to the haptoglobin-CD163 haemoglobin scavenging system active in human SAH [25]) as well as a range of antioxidative and detoxifying enzymes. There was a resultant reduction in oxidative damage and inflammation in brain areas endangered by the intraparenchymal hematoma. Neutrophil count, oxidative damage, and behavioural deficit were shown to be reduced in intracerebral haemorrhage-affected brain tissue of the SFN treatment group.

Furthermore, Nrf2-deficient mice demonstrated more severe neurologic deficits after intracerebral haemorrhage and did not benefit from the protective effect of SFN. Therefore, activation of Nrf2 with SFN after intracerebral haemorrhage may represent a potential target for combating associated damage [16] or conditions with similar pathogenesis such as SAH.

The protective role of SFN in intracerebral haemorrhage was replicated in a further study investigating the role of haptoglobin after SAH. Haptoglobin is a plasma protein that binds cell free haemoglobin with high affinity. This has been demonstrated to be active in human SAH. In rodents SFN upregulated haptoglobin expression and alleviated intracerebral haemorrhage mediated brain injury [26].

SAH makes a better target for SFN treatment given blood is distributed over the entire surface of cortex resulting in a more severe inflammatory response. This is supported by the observation that there is a higher rate of late secondary deterioration and poor outcome after SAH than in intracerebral haemorrhage. The effect of SFN in SAH has been investigated *in vitro* using vascular smooth muscle cultures from rat aorta exposed to oxyhaemoglobin as a model for SAH. In this model Nrf2 is up-regulated; the effect is increased in the SFN group compared to the control group, whereas the increase in the inflammatory cytokines (IL-1b, IL-6 and TNF-α) observed 48 h after oxyhaemoglobin treatment, is markedly reduced by SFN [11].

The effect of SFN was also investigated in an *in vivo* rat SAH model with intraperitoneal SFN injections 30m, 12 h and 36h after SAH. 0.3 ml fresh arterial, nonheparinized blood into the

prechiasmatic cistern was injected over 20 sec. As a result, Nrf2 and its target gene product, haeme oxygenase-1 (HO-1), were upregulated in the cortex after SAH and peaked at 24 hr post-SAH. After intraperitoneal SFN administration, the elevated expression of Nrf2-antioxidant response element related factors such as Nrf2, HO-1, NAD(P)H:quinone oxidoreductase 1 (NQO1), and glutathione S-transfer- ase-a1 (GST-a1) were detected in the cortex at 48 hr following blood injection. In the SFN treated group, early brain damage such as brain oedema, blood–brain barrier impairment, cortical apoptosis, and motor deficits were significantly ameliorated compared with vehicle- treated SAH rats [19].

1.3. Risks / Benefits

The potential therapeutic benefits of SFX-01 appear to outweigh any potential risks for patients with SAH who may receive it with the intent of reducing DCI. SFN has been demonstrated to be a potent activator of cellular oxidative stress defence mechanisms via activation of Nrf2 and to initiate over-expression of HO-1 and NQO1, both enzymes responsible for maintenance of cellular oxidative balance. This pathway has been demonstrated in the literature to be of critical importance in neuroprotection. These protective effects have been demonstrated with the active component of SFX-01, SFN, in both *in vitro* and *in vivo* animal models of cerebral ischemic disease and SAH. .

In vivo PK studies demonstrate similar PK properties for SFN when delivered from SFX-01 or synthetic SFN itself. As such, we believe that SFX-01 has the potential to offer significant therapeutic benefit for patients with SAH.

In pre-clinical toxicology and safety pharmacology studies of SFX-01, the principle toxicities were GI disturbance, which in dogs was manifested as severe vomiting, precluding their use as a study species. The only other toxicity of note was diffuse urothelial hyperplasia. This was observed in both rat and primate 4-week studies; in the rat, at all doses (i.e. No-Observed-Adverse-Effect level (NOAEL) was undetermined), in the primate, a NOAEL of 65 mg/kg/day was identified. There was also mild epithelial hyperplasia observed in the stomach and small intestine of the rats but not primates, which recovered during the no-treatment period. The significance of this finding in the urothelium is not clear. It has been reported in the rat with a number of other compounds, such as phenacetin, penem antibiotics and sodium saccharin. In no cases did these findings occur in man.

SFN, the active component of SFX-01, has been extensively studied in man when delivered from botanical precursors e.g. myrosinase-activated glucoraphanin. Exposures of 1.3ug/ml (7.4µM) with Area Under the Curve (AUC) of 7.1ug.h/ml (7100ng.h/ml) have been reported in man. These doses produced only mild gastro-intestinal (GI) disturbances and no other significant toxicities [27]. Experience with broccoli sprout extracts, glucoraphanin and SFN in healthy volunteers and patients demonstrated that the associated principle adverse events were self-limiting mild GI disturbance. No significant symptomatic, biochemical or electrophysiological changes were reported.

Note that previous human experience with SFN or precursors to SFN, whilst reported in the literature, may not provide wholly convincing safety information since SFN exposures may have been significantly lower than will be achieved with SFX-01 dosed at 300mg bid.
Given this, the study has been designed with an explicit interim safety assessment by an independent Data Safety Monitoring Board (DSMB) after 20 patients have been dosed (randomised to SFX-01 or placebo) as inpatients. Note also that there is no female human experience with SFX-01; therefore gender difference in kinetics and/or tolerability have not been explored.

In initial clinical trials with SFX-01 (in 12 healthy males) there was no evidence of adverse biochemical or haematological effects in up to a single dose of 700 mg (EVG001N) and repeat (7 days) doses of 600 mg once daily or 300 mg bid (EVG002N).

The compound was well tolerated up to 300 mg but thereafter mild signs of GI effects were observed. Evgen Pharma plc believes that these are due to release of the active pharmaceutical ingredient in the stomach and have now developed an acid resistant capsule formulation to allow the dose to pass directly into the lower GI tract before release. These mild AEs were ameliorated by the acid resistant product and with food even at 600 mg doses.

The exposure in man at the top dose of 700 mg single dose and 600 mg repeat dose remained below those that produced any adverse effects in the primate repeat dose toxicology study. (Primate pharmacokinetics (PK) showed Mean Cmaxs at a dose of 65mg/kg/day of 209ng/ml on day 1 and 135ng/ml on day 28 with mean Areas Under the Curve of 498ng.h/ml on day 1 and 729ng.h/ml on day 28)

Daily doses of 600mg per day in man delivered SFN systemic exposures equivalent to the NOAELs seen in the primate repeat dose toxicology with SFX-01- i.e. there are no margins to the NOAEL. However, for the anticipated therapeutic dose (300 mg SFX-01 bid), the mean Cmax values for sulforaphane plasma concentration ranged between 81.63 ng/mL to 123.24 ng/mL. Median Tmax was found to occur between 1.00-3.00 hours and AUC 0-12 hours ranged from 244.06 ng·hr/ml to 306.09 ng·hr/ml.

The proposed clinical study in man involves daily doses of SFX-01 in patients with SAH at a dose of 300 mg bid SFX-01, (equivalent to a daily dose of 92 mg SFN). The potential benefit to patients is significant with reduced DCI, improved cognition and cerebral function. The risk is modest, with exposures expected to remain below those shown to produce any adverse effects in primates.

In summary, the preclinical data supports the premise that SFX-01 offers significant potential therapeutic benefits, without significant clinical toxicities. The adverse event profile of the compound in primates and Phase I clinical studies is modest; there is a significant margin between the proposed dose in man and that producing any toxicities in primate-repeat dose toxicity studies. As such, studies in patients with SAH are warranted, with a view to defining the clinical benefits and overall efficacy profile.

1.4. Study Rationale

This is a randomised, double-blind, parallel-group study comparing SFX-01 (300 mg) taken orally as capsules or as a suspension via a nasogastric tube (NG) twice-daily for up to 28 days versus placebo in 90 patients who have had SAH and present within 48 hours of ictus. The treatment group will receive SFX-01 in order to improve and reduce the long-term complications of SAH such as DCI, as reflected by Trans-Cranial Doppler (TCD) readings. The objective is to demonstrate safety and tolerability and search for pharmacokinetic and pharmacodynamic signals in patients that have suffered a SAH. The results of the study will be used to design adequately powered efficacy studies with defined clinical endpoints.

1.5. Dose Rationale

Animal studies in ischaemic stroke, intracerebral haemorrhage and subarachnoid haemorrhage have all used doses of 5mg/kg of sulforaphane in rodents [19, 22, 24, 28]. Conversion of this dose to humans following body surface area as has been widely recommended [29, 30] and yields an effective dose of SFN in humans of 50mg. This is equivalent to 300 mg of SFX-01 (300 mg of SFX-01 contains 46.15 mg of SFN).

In the clinical studies conducted to date, SFX-01 has been shown to be well tolerated up to 300 mg twice-daily with no serious adverse effects.

1.6. Trial Conduct

This study will be conducted in compliance with the protocol and according to Good Clinical Practice and applicable regulatory standards. No deviation from the protocol will be implemented without the prior review and approval by the relevant ethics and regulatory authorities, except where it may be necessary to eliminate an immediate hazard to a research subject. In such case, the deviation will be reported to the relevant ethics and regulatory authorities as soon as possible.

1.7. Population

The population to be studied are patients with spontaneous aneurysmal SAH, Fisher grade 3 or 4 on CT, who present within 48 hours of ictus.

2. STUDY OBJECTIVES AND ENDPOINTS

2.1. Primary Objective

Safety

To evaluate the safety of up to 28 days of SFX-01 dosed at up to 92mg Sulforaphane (SFN) per day.

Pharmacokinetic

To detect the presence of SFN in Cerebrospinal Fluid (CSF).

Efficacy

To determine if a minimum of 7 days treatment with SFX-01 reduces Middle Cerebral Artery (MCA) peak flow velocity following subarachnoid haemorrhage (SAH).

2.2. Secondary Objectives

The secondary objectives are:

- To determine if a minimum of 7 days treatment with SFX-01 improves clinical outcome following SAH as measured using the modified Rankin Scale assessed at 7 days, discharge, 28, 90 and 180 days post ictus.
- To determine blood SFN levels (and its metabolites) with treatment with SFX-01 (300mg bid).
- To determine CSF SFN levels and kinetics with treatment with SFX-01 (300mg bid).
- To determine if up to 28 days treatment with SFX-01 increases blood haptoglobin (HP) levels and decreases malondialdehyde (MDA) levels following SAH.
- To determine if up to 28 days treatment with SFX-01 can reduce the incidence of Delayed Cerebral Ischaemia (DCI) following SAH.
- To determine if up to 28 days treatment with SFX-01 improves long-term outcome in subjects following SAH.
- To determine if up to 28 days of treatment with SFX-01 can reduce iron deposition and cortical atrophy following SAH.

2.3. Primary Outcome Endpoints

The primary outcome endpoints are:

Safety

- Concomitant medication
- Adverse events
- Change in grading of AE severity
- FBC, U&Es, LFT, CRP & Urine Microscopy
- INR or PT, APTR or APTT, & Fibrinogen (Clauss or Derived)at 7 & 28 days

Pharmacokinetic

- Presence of SFN (or its metabolites) in CSF

Efficacy

- The maximum Middle Cerebral Artery (MCA) velocity determined using Trans-Cranial Doppler (TCD)

2.4. Secondary Outcome Endpoints

The secondary outcome endpoints are:

- Modified Rankin Scale (mRS), at 7 days, discharge, 28, 90 and 180 days.
- Incidence of Delayed Cerebral Ischaemia (DCI) defined as a new focal deficit or reduction in Glasgow Coma Scale (GCS) ≥2 if not explained by other causes (i.e. re-bleed, hydrocephalus, seizure, meningitis, sepsis or hyponatraemia)
- Incidence of new cerebral infarct on Computed Tomography (CT) or Magnetic Resonance Imaging (MRI).
- Institution of hypertensive (triple H) therapy for presumed DCI
- SF-36 quality of life survey at 28, 90 & 180 days.
- Checklist for Cognitive and Emotional Consequences (CLCE-24), Brain Injury Community Rehabilitation Outcomes Scale (BICRO-39) at 90 & 180 days.
- Subarachnoid Haemorrhage Outcome Tool (SAHOT) and Glasgow Outcome Scale – Extended (GOSE) at 28, 90 & 180 days.
- Length of acute hospital stay
- Discharge location
- Amount of iron identified on MRI Susceptibility Weighted Imaging (SWI) 180 days after start of treatment.
- Cortical atrophy on T1 MRI at 180 days after start of treatment

Non-EVD Patients: i.e. patients will have a Lumbar Puncture for Collection of CSF

- Blood HP and MDA, Proteomic & Genomic concentration at baseline (pre dose 0-48 hours), D7 and D28
- Paired $CSF_{(Lumbar\ Puncture)}$/blood HP, MDA, Proteomic & Genomic & SFN/SFN metabolite concentration at Day 7

EVD Patients: i.e. Will not have a lumbar puncture

- Blood HP, MDA, Genomic & Proteomic concentration at baseline (pre dose 0-48 hours), D7 and D28

- Paired $CSF_{(EVD)}$/blood HP, MDA & Proteomic/Genomic concentration on alternate days (+/- 1 day) starting from day of EVD fitting to D14 or until EVD removal.
- Paired $CSF_{(EVD)}$/blood HP, MDA, Proteomic/Genomic & SFN/SFN metabolite concentration at day 7

Subset of 12 EVD Patients: In addition to all other all other sampling the following samples will be taken:

- Serial paired $CSF_{(EVD)}$/blood SFN/SFN metabolite concentration at one of the first 3 doses and at day 7

3. STUDY DESIGN

3.1. Overall Study Design and Plan Description

This is a Safety, Tolerability, Pharmacokinetic and Pharmacodynamic Study of SFX-01 in Subarachnoid Haemorrhage with exploratory evaluations of efficacy.

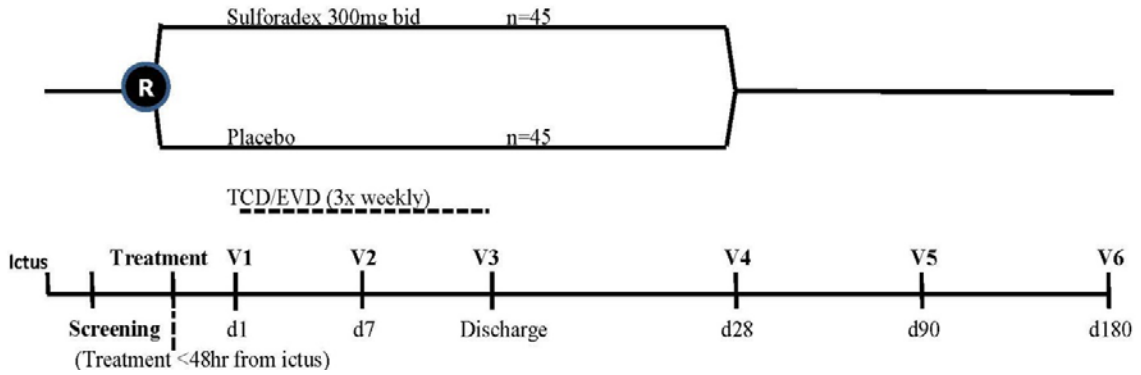
The study is a randomised, double-blind, parallel-group design comparing SFX-01 (300 mg) taken orally as capsules or as a suspension via a nasogastric tube (NG) twice-daily for 28 days versus placebo in at least 90 patients who have had SAH and present within 48 hours of ictus.

The treatment group will receive SFX-01 in order to improve and reduce the long-term complications of SAH such as DCI, as reflected by TCD readings. The objective is to demonstrate safety and search for signals of efficacy in patients that have had a SAH.

A sub-study will be conducted in up to 12 patients with an External Ventricular Drain (EVD) fitted; serial CSF samples will be taken pre- & post-dose on two occasions to determine pharmacokinetics of SFN in CSF in comparison with plasma pharmacokinetics. Substudy patients will undergo all other procedures. Initial treatment duration will be for the length of time participants remain an inpatient in tertiary care (up to day 28 post ictus dosing) followed by treatment up to day 28 post ictus (including post-discharge); follow up duration is 28 days, three and six months. The planned trial period is 24 months.

The Per Protocol Population (for Primary analysis) will be considered to be those patients that have been dosed for a minimum of 7 days and for whom it can be shown that there were no dispensing errors.

Flowchart



3.2. Duration of Treatment

Initial duration of treatment (for the first 20 patients) will be limited to the length of time they remain an inpatient in tertiary care (up to a maximum of day 28 post ictus). Direct supervision will be guaranteed during the acute inpatient stay in the neurological centre; patients will not be discharged with the investigational medicine.

Once 20 patients have completed dosing up to day 7 post ictus, a DSMB will convene to review the data; a decision will be made as to dosing after discharge from tertiary care.

The final duration of treatment is intended to be up to day 28 post ictus. However, as the sizable group of patients will return to their local hospital or rehabilitation units for further care, on discharge they will be supplied with the medication for the remainder of the treatment together with instruction for the discharge destination. If patients are to be discharged home, they or their carer will be given clear instruction on how to continue with the treatment and the need to store the IMP at the correct refrigerated storage temperature of between 2-8°C. In addition to detailed instructions a medication compliance diary will be provided on discharge and collected at Day 28 together with any remaining study medication for reconciliation.

The definition of "discharge" can change from site to site, therefore, for the purposes of this trial discharge is where the consultant responsible for the intervention of treating the SAH decides that their specialist care is no longer needed and they can safely transfer that care to another health care professional or send the patient home. Even if the patient remains in the same unit they will still be deemed to be discharged. The timing to the next assessment in the schedule should start when this decision has been made.

3.3. Premature Discontinuation of Treatment

Patients have the right to discontinue trial medication at any time and for any reason. The Investigator also has the right to discontinue trial medication if they feel that treatment is no longer appropriate, if in their opinion the patient's clinical condition is worsening or for safety (adverse events).

Patients removed from treatment will be encouraged to continue in the study and complete the study visits in accordance with the study visit schedule.

Investigators may discontinue a participant from the trial at any time if they encounter any of the exclusion criteria as well as:

- Significant non-compliance with treatment regimen or trial requirements
- An adverse event which requires discontinuation of the trial medication or results in inability to continue to comply with trial procedures
- Disease progression which requires discontinuation of the trial medication or results in inability to continue to comply with trial procedures
- Lost to follow up

If a patient prematurely discontinues treatment the reason for discontinuation will be recorded on the electronic Case Report Form (eCRF). Unless the patient withdraws consent for the use of data, any data from a discontinued patient will still be utilised for study analysis.

3.3.1. Cessation of Treatment

A patient will be classified as having ceased treatment when he or she discontinues medication, prior to the completion of the prescribed course for any of the following reasons:

- Adverse Event

- Death
- Lost to follow up
- Withdrawal of consent
- Protocol Violation
- Pregnancy
- Treatment is no longer appropriate
- Termination of trial

3.3.2. Withdrawal from Trial

Patients have the right to withdraw from the trial at any time and for any reason. If a patient refuses to be seen for further visits the assessments for discharge or Day 28 will be performed at the time they have indicated that they will not attend for further visits, assuming they are available and consent to this.

3.3.3. Replacements

In order to provide reliable data from at least 90 patients who meet the per protocol requirements, additional patients may be recruited to replace individuals when there is evidence either that insufficient study medication has been taken or that there might have been errors in study medication dispensing.

3.4. Discussion of Study Design, Including the Choice of Control Groups

The study has a parallel group design, which is deemed more appropriate than a cross-over design considering the aetiology of the illness. Patients will be randomised, in equal proportions, to placebo or the active dose regimen (SFX-01 300 mg capsule taken orally twice-daily for 28 days).

The only effective treatment to reduce morbidity from SAH is nimodipine [4]. However, its effects are small and despite its use poor outcomes remain a significant problem as evidenced by contemporary outcome data since its introduction [5]. Moreover, even in the survivors conventionally considered to have made a good recovery, neurocognitive deficits are common leading to extensive problems with social reintegration and functioning in the workplace [6]. For this reason SFX-01 is compared to placebo only. However, SFX-01 will be used in conjunction with nimodipine as per routine clinical care. The study is randomised in order to prevent bias in the allocation of treatment and to ensure the comparability of baseline characteristics between the treatment groups. In order to prevent bias in the conduct of the clinical assessments, the study is double blind, so that neither the investigators nor the patient know whether the patient is receiving active treatment or placebo.

4. SUBJECT SELECTION CRITERIA

4.1. Subject Recruitment

All patients, admitted with a diagnosis of spontaneous SAH will be assessed by the study team against the inclusion and exclusion criteria. Identified subjects who fulfil the criteria will then be approached by a member of the research team on the delegation log, who will in turn obtain consent from the subject, the Personal Legal Representative or Professional Legal representative in the case of adults lacking capacity if possible (see section 14).

The Patient Information will be given to the subject, Personal Legal Representative or Professional Legal representative; they will be given sufficient time in order to make a decision.

In the case of adults lacking capacity where no Personal Legal Representative or Professional Legal representative is available patients may be randomised and receive the first two doses whilst consent is being sought. (***Note that this is only permissible where local regulations allow; if local regulations do not allow emergency dosing without consent the patient shall not be enrolled into the study.*** see section 14).

4.2. Inclusion Criteria

1. Patients with radiological evidence of spontaneous aneurysmal SAH
2. Fisher grade 3 or 4 on CT
3. Definitive treatment of aneurysm has not been ruled out
4. Previously living independently
5. In the opinion of the investigator, the delay from ictus to randomisation and initiation of trial medication will not exceed 48 hours
6. Aged 18 to 80 years
7. In the opinion of the investigator it will be possible to obtain Informed Consent from the Patient, Personal Legal Representative or Professional Legal representative within 24 hours of first dose

4.3. Exclusion Criteria

1 Traumatic SAH
2 Fisher grade 1 or 2
3 SAH diagnosed on lumbar puncture with no evidence of blood on CT
4 Decision not to treat aneurysm has been made
5 Plan to withdraw treatment
6 Significant kidney disease as defined as plasma creatinine ≥2.5mg/dL (221 μmol/l)
7 Liver disease as defined as total bilirubin ≥2-fold the upper limit of normal as measured by the local laboratory
8 Females who are pregnant or lactating.
9 Participants enrolled in another interventional research trial in the last 30 days
10 Patients for whom it is known, at the time of screening, that clinical follow-up will not be feasible
11 Patients unwilling to use two forms of contraception (one of which being a barrier method see section 7.9) 90 days for men and 30 days for women after last IMP dose
12 Known hypersensitivity to any component of a sulforaphane containing product including broccoli

4.4. Subject Withdrawals

In accordance with Informed Consent and the Declaration of Helsinki, the patient may discontinue the study at any time without giving any reason.

In all circumstances, Patients or Legal Representatives will be made aware of the rights to refuse participation in a clinical trial and will be entitled to freely withdraw their informed consent, without giving reasons. Patients or Legal Representatives should be assured that their withdrawal from the trial will not cause prejudice, will not result in any detriment and will not affect their treatment. In addition, refusal to give consent or withdrawal of consent to participate in research will not lead to any liability or discrimination (e.g., with regard to insurance or employment) against the person concerned.

In addition, the Investigator also has the right to withdraw subjects from the study for any reason.

Should a Patient and/or Legal Representative decide to withdraw for other reasons, all efforts will be made to complete and report the observations as thoroughly as possible. A complete final evaluation at the time of withdrawal will be performed with an explanation of the reason why the subject is withdrawing from the study.

The Investigator is responsible for the optimal individual treatment of the patient.

The Investigator must fill in the "Study termination" section in the eCRF describing all reasons for withdrawal.

After a patient withdraws from the trial, the Investigator remains responsible for reporting SAEs considered causally related to the study drug. In addition, the Investigator needs to ensure appropriate treatment and follow-up of each adverse event still ongoing at the time of the patient's discontinuation.

4.4.1. Replacement of Withdrawn Patients

In the instance where a patient has been entered into the trial prior to informed consent being obtained (i.e. through the emergency consent procedure) and consent is subsequently refused or not obtained within 24 hours by the patient and/or legal representative, the participant shall be withdrawn and replaced.

Replacement of patients who withdraw/are withdrawn prior to completion of day 7 (post ictus) is to be discussed (by the Investigator and Sponsor) and approved by the Investigator and Sponsor's Medical Monitor on a case by case basis.

Patients who withdraw for any other reason after randomisation will not be replaced.

Unless the patient withdraws consent for the use of data, any data from a discontinued patient will still be utilised for study analysis.

5. CONCOMITANT MEDICATIONS

Concomitant treatment will be permitted unless its use is contraindicated or there are significant interactions with SFX-01.

SFX-01 is contraindicated in those who are hypersensitive to any component of a SFN-containing product, including broccoli.

5.1. Permitted Concomitant Medications

In the context of aneurysmal SAH, all patients will also receive nimodipine 60 mg every four hours or 30 mg every two hours.

5.2. Non-Permitted Concomitant Medications

There are no known contraindicated medicines that have a significant interactions with SFX-01.

6. TREATMENTS

6.1. Appearance and Content

SFX-01 (active 300 mg capsule) and placebo (placebo 300 mg capsule) will be taken orally or via a naso-gastric tube twice-daily for 28 days.

6.2. Dosage and Administration

6.2.1. Trial Drug

Name: SFX-01

Presentation: Size 00 acid resistant Hydroxypropyl Methycellulose (HPMC) capsules (White OP) providing 300 mg SFX-01

6.2.2. Placebo

Identical size 00 acid resistant HPMC capsules containing 300 mg alpha cyclodextrin.

6.2.3. Administration

Capsules will be swallowed whole with water.

In patients who are unable to take tablets orally but have a NG tube in situ the study drug will be administered via the tube. The capsule will be opened in the neurointensive care or neurosurgical ward and emptied into 20ml water.

The trial medication will be administered to the patient twice-daily while an inpatient at the neurosurgical centre. The trial medication will be dispensed to the patient at discharge (once the 20 patient DSMB has agreed post-discharge dosing).

6.2.4. Dose Frequency De-escalation

In the event of tolerability problems whilst the patient is in the neurosurgical centre, the Investigator will assess whether simple measures to ease the effects of the adverse event(s) may be implemented (for example ant-acid in the case of GI irritation or anti emetic in the event of nausea).

The investigator will also assess whether or not the adverse event(s) could be related to the trial medication and severe enough to warrant a dose frequency reduction.

In the first instance the investigator may consider missing one dose.

If a dose frequency reduction is warranted, from that point onwards the second daily dose will be omitted; a dose frequency increase back to twice daily will not be permitted.

If tolerability problems continue then investigational medication will be stopped; patients will continue in the study and complete the study visits in accordance with the schedule of assessments.

The staged dose frequency de-escalation (dropping to once daily) will not be carried out after discharge from the neurosurgical centre; if tolerability problems occur after discharge dosing with the investigational medication will be stopped; patients will continue in the study and complete the study visits in accordance with the schedule of assessments.

6.3. Packaging & Storage

IMP is assembled for Qualified Person Certification and release for use in the clinical trial at the contract manufacturing company (PharMaterials) and delivered to sites at refrigerated conditions of between 2-8^{o}C as required. The bulk manufactured IMP is placed into 60 ml Duma high-density polyethylene bottles in compliance with EU Directive 2002/72/EC and FDA title 21 CFR §177.1520 and closed with Duma 45 mm round plastic tamper-evident screw

cap with three breakpoints on the tamper-evident ring and integrated silica gel desiccant in compliance with the specification. Each bottle contains 56 size 00 capsules.

IMP is to be stored in refrigerated conditions of between 2-8^{o}C at all times in the pharmacy with a limited number of bottles to be stored securely and in refrigerated conditions of between 2-8^{o}C on the Neurointensive Care Unit, with access restricted to authorised personnel. When a patient is randomised, the next lowest numbered bottle within the specified strata will be utilised from the stock. A member of the research team will place the patient's name, date of birth and trial number on the bottle; it will be stored on the ward and be discharged with the patient.

Upon discharge from hospital the patient will be provided with a cool bag containing a refrigerated gel pouch for transportation of the IMP to home, or transfer to another hospital where upon the IMP will then be stored at the protocol required temperature of between 2-8°C at all times.

6.4. Labelling

All study medications will be labelled in accordance with Annex 13: Manufacture of investigational products.

Study medication packs will be labelled identifying the site and strata according to the WFNS grading scale score.

6.5. Blinding and Randomisation

6.5.1. Randomization

Patients will be allocated to double-blind medication through a stratified randomisation schedule with the strata defined by site and by baseline severity defined by WFNS score of 1-3 or 4 & 5.

All treatment packs will be otherwise identical in appearance, in order to maintain patient and investigator blinding throughout the trial. The contents will also be indistinguishable should they be opened either inadvertently or for the purposes of NG administration. Patients will be randomised to one of treatment groups by allocation of the appropriate, numbered, treatment pack. The treatment packs will be pre-numbered according to a block balanced randomisation code generated by PharMaterials.

Emergency code envelopes will be produced to provide details of the medication regimes each patient has been allocated to. Sealed code break envelopes will be held by the Pharmacy and by Diamond Pharma Services.

6.5.2. Blinding

<u>Code Breaks at the Trial Centre/ Diamond Pharma Services.</u>
The Pharmacy will receive a sealed envelope containing the identity of each trial medication bottle dispensed during the trial. An envelope may be opened only in the case of a serious adverse event and only when it is essential to the subsequent management of the patient. Diamond Pharma Services will be responsible for breaking codes for regulatory submissions of Suspected Unexpected Serious Adverse Reactions (SUSARs), thereby maintaining the overall confidentiality of the code breaks.

Where a code break has occurred, the Investigator must provide a written record of the circumstances surrounding the event but should take care not to record, on any trial

documentation, details of the unblinded treatment. Such details should be disclosed only to those persons who have responsibility for the immediate management of the patient. The Clinical Trial Company (TCTC) must be notified as soon as possible. If the code is broken the data for that patient will be excluded from the Per Protocol Population analysis but included in the Intention to Treat Analysis. They will continue in the study and complete the study visits in accordance with the study visit schedule.

At the end of the treatment phase of the trial, the envelopes must be returned, along with the drug dispensing records to the trial monitor. The envelopes will be checked to ensure that the seals have not been broken unless (unless a code break has been carried out). Emergency code envelopes may only be opened in an emergency, when the patient's condition requires knowledge of the test medication. Every attempt should be made to ensure that all persons directly involved in the trial remain ignorant of the randomisation codes. The Safety Committee will be able to request unblinding of patients (see section 25).

The randomisation code will not be fully revealed, other than in instances where code break is justified on grounds of safety, until all data have been gathered, entered into the database, clarified, resolved, verified, validated and the database has been closed. The code will then be broken by the statistician. The Investigator will be advised of allocation of trial treatment following communication of the results of the analysis.

Prevention of unblinding by laboratory measurements
Laboratory data for blood/CSF SFN levels will be entered into a separate database by a member of the research team. The data from the laboratory database will be transferred electronically to the main database.

Treatment compliance
Compliance with treatment will be recorded during the inpatient hospital stay by health care professionals and/or a member of the research team. On discharge to the usual residence this responsibility is to be carried out by the patient or their Personal Legal Representative, aided by detailed instructions.

In the event of discharge to a rehabilitation unit or patient local hospital, written instructions will be given to the patients on discharge and verbal communication with the clinical team will be made to ensure compliance.

All patients will be discharged with a patient diary which will be filled in and collected at Day 28.

Compliance will be further monitored by drug reconciliation. Patients will be asked to return the medication bottle and any residual contents at the Day 28 visit. At this time any residual tablets will be counted and recorded.

6.6. Drug Storage

All medication supplied in connection with the trial will be used only for this and no other purpose.

IMP will be stored under refrigerated conditions of between 2-8° C at all times in the Pharmacy, hospital wards and also upon discharge to another hospital and the patient's home. A cool bag containing a refrigerated gel pouch will be provided for transportation purposes.

IMP is to be stored in the pharmacy with a limited number of bottles to be stored securely on the Neurointensive Care Unit.

6.7. Drug Accountability

All members of the research team, including investigators are accountable for the supply of the medication during patients' hospital stay. This requires the keeping of records of dispensing medication, as well as inventory checks. All members of the study team will adhere to local guidelines in addition to GCP. The study drug will only be used in those who are enrolled on to the study and for the named patients only.

Record keeping including delivery of medication to pharmacy and wards, dispensing to the subject, unused study medication and return of unused medications will be continuously monitored and updated. Study medication will be prescribed on the drug chart and the nursing staff and/or study team involved will keep daily records of its administration.

7. STUDY PLAN

7.1. Continuous Assessments:

The following assessments will be carried out on a continuous basis and as events arise:

- Adverse Events
- Concomitant Medication
- Medication Compliance

7.2. Pre-Dose Assessment (within 48 hours of ictus):

The following assessments will be performed and documented in all patients admitted with a diagnosis of spontaneous aneurysmal SAH:

- Informed Consent (within 24 hours of first dose)
- Inclusion/Exclusion (screen failures to be recorded)
- Recording results of CT/MRI & Fisher grading (Required for SAH diagnosis)
- Demographics/Medical History
- Physical Examination
- Pregnancy test - urine or blood is acceptable
- Concomitant Medication,
- Protocol specific Safety Bloods and Urine (Biochemistry, Haematology, Lipid Profile & Coagulation Status - see Section 8.1). If no predose safety urine is available, then baseline urine taken prior to day 2 dosing will be acceptable.
- Blood HP, MDA, Proteomic/Genomic sampling
- Best World Federation of Neurological Societies (WFNS) scale at first presentation, on admission to the neurosurgical unit and after resuscitation (when this should occur)

For patients lacking capacity where a Personal Representative is not immediately available in person, a Professional Legal Representative will be sought. If they are in attendance in person they will discuss the trial with the research team and complete a consent form if they feel it is appropriate for the subject to participate in the trial.

If they are not in attendance in person they will be contacted by telephone and their opinion sought. If in agreement, the study team will document this in the patient notes (details of the representative, date and time of the telephone call, summary of the discussion and Informed Consent process and version of the Informed Consent Form), the patient will be enrolled and the Professional Legal Representative will complete a consent form the next time they attend the patient.

For patients lacking capacity where a Personal Legal Representative was not immediately available, written informed consent will be obtained and documented from the Personal Legal Representative at the earliest opportunity.

For patients lacking capacity at screening, with informed consent obtained from their Personal Legal Representative or Professional Legal Representative, written informed consent will be obtained and documented from the patient as soon as they regain consciousness sufficiently to do so.

In the case of adults lacking capacity where no Personal Legal Representative or Professional Legal representative is immediately available (including Professional Legal Representative unavailable by telephone) patients may be randomised and receive the first two doses whilst consent is being sought. (***Note that this is only permissible where local regulations allow; if local regulations do not allow emergency dosing without consent the patient shall not be enrolled into the study.)***

In the instance where a patient has been entered into the trial prior to informed consent being obtained (i.e. through the emergency consent procedure) and consent is subsequently refused or not obtained within 24 hours by the patient and/or legal representative, the participant shall be withdrawn and replaced (see section 14).

If the patient has not regained capacity by the time of the 180 Day follow up no further attempts will be made to obtain consent directly from the patient.

7.3. Dose (within 48 hours of ictus)

Eligible patients will be randomized to either trial drug (SFX-01 300 mg) or placebo. The first administration of trial medication must not exceed 48 hours from ictus.

Patients are to be dosed with study drug (SFX-01 or placebo) twice daily, approximately 12 hours apart, until Day 28 post ictus.

7.4. Post Dose (12-24 hours after first dose)

The following procedure will be carried out after the first dose:

- Protocol specific safety blood tests (see Section 8.1)

7.5. Between Pre-dose Assessment & Post Dose:

The following assessments are to be carried out pre or post dose.

- Recording of results and form of angiographic assessment by Computed Tomography Angiography/Digital Subtraction Angiography/Magnetic Resonance Angiography
- First Trans-Cranial Doppler reading (to measure peak MCA flow)

The Angiographic Assessment (CTA/DSA/MRA) and procedure planned and carried out (clipping/coiling) is to be recorded

7.6. Ongoing Assessments – All patients:

The following procedures and assessments are to be carried out on alternate days until no longer clinically indicated:

- Safety Bloods as part of normal standard clinical care

- TCD readings (to be performed on alternate days (± 1) post ictus (starting day 3 (± 1) until at least Day 7 (± 1) or discharge whichever is sooner. Any additional TCD readings obtained after this point will also be recorded)
- Glasgow Coma Score

7.7. Patients with an External Ventricular Drain fitted: Ongoing EVD single sampling (on alternate days +/- 1day from day of EVD fitting until D14 or until the EVD is removed):

Patients with an External Ventricular Drain (as part of standard of care) will have the following procedures and assessments carried out on alternate days (±1) from the day of EVD fitting until removal:

- An alternate day single EVD CSF sample for determination of HP & MDA levels
- An alternate day single blood sample for HP, MDA & Proteomic/Genomic analysis

The samples are to be taken at the same time (paired sample).

7.8. Day 7 post ictus (± 1 day):

The following procedures and assessments are to be carried out at Day 7 post ictus:

- Protocol specific Safety Bloods and Urine (see Section 8.1)
- TCD reading
- modified Rankin Scale
- Concomitant Medication Review

Non-EVD patients only:

- Paired blood Sampling and lumbar puncture to determine CSF/blood HP, MDA, Proteomic /Genomic & SFN/SFN metabolite concentrations

EVD patients only:

- Paired blood and EVD sampling to determine CSF/blood HP, MDA, Proteomic /Genomic & SFN/SFN metabolite concentrations

7.9. Within 2d of discharge:

The definition of “discharge” can change from site to site, therefore, for the purposes of this trial discharge is where the consultant responsible for the intervention of treating the SAH decides that their specialist care is no longer needed and they can safely transfer that care to another health care professional or send the patient home. Even if the patient remains in the same unit they will still be deemed to be discharged. The timing to the next assessment should start when this decision has been made.

The following procedures and assessments are to be carried within two days of discharge from the neurosurgical unit:

- Protocol specific Safety Bloods and Urine (see Section 8.1)
- modified Rankin Score
- Glasgow Coma Score

If the outcome of the 20 patient DSMB has determined that post-discharge dosing is acceptable, the patient will be discharged with sufficient medication to ensure full-compliance until Day 28

post-ictus. If patients are to be discharged home they or their Personal Legal Representative will be given clear instruction on how to continue with the treatment including the requirements of refrigerated storage conditions of between 2-8°C at all times. In the event of discharge to a rehabilitation unit or hospital, written instructions will be given to the patients on discharge and verbal communication with the clinical team will be made to ensure compliance. All patients will be discharged with a treatment compliance sheet (diary) which will be filled in and collected at Day 28 post ictus.

The patient will be reminded to use two forms of contraception (one of which being a barrier method) for 90 days for men and 30 days for women (see Contraceptive Requirements)

The patient's GP will also be informed of the patient's discharge and entry in to the clinical trial. If discharged to home, the patient, or their carer, will receive a follow-up phone call within 2 days following discharge.

Contraceptive Requirements:

Male subjects

Male subjects whose female partner(s) is (are) pregnant must use a condom from the time of the first administration of treatment or study medication until three months (90 days) following administration of the last treatment or dose of study medication.

If the subject has undergone surgical sterilisation (vasectomy with documentation of azoospermia) a condom must be used.

Male subjects must use acceptable methods of contraception if the male subject's partner could become pregnant from the time of the first administration of treatment or study medication until three months following administration of the last treatment or dose of study medication. The acceptable methods of contraception are as follows:

- Condom and occlusive cap (diaphragm or cervical/vault caps) with spermicidal foam/gel/film/cream/suppository
- Surgical sterilisation (vasectomy with documentation of azoospermia) and a barrier method (condom or occlusive cap (diaphragm or cervical/vault caps) used with spermicidal foam/gel/film/cream/suppository)
- The female partner uses oral contraceptives (combination oestrogen/ progesterone pills), injectable progesterone or subdermal implants and a barrier method (condom or occlusive cap (diaphragm or cervical/vault caps) used with spermicidal foam/gel/film/cream/suppository)
- The female partner has undergone documented tubal ligation (female sterilisation). In addition, a barrier method (condom or occlusive cap (diaphragm or cervical/vault caps) used with spermicidal foam/gel/film/cream/suppository) must be used
- The female partner has undergone documented placement of an intrauterine device (IUD) or intrauterine system (IUS) and the use of a barrier method (condom or occlusive cap (diaphragm or cervical/vault caps) used with spermicidal foam/gel/film/cream/suppository)
- True abstinence when this is in line with the preferred and usual lifestyle of the subject. *Periodic abstinence (e.g., calendar, ovulation, symptothermal, post-ovulation methods) and withdrawal are not acceptable methods of contraception*

Female subjects

Female subjects of childbearing potential must use medically acceptable methods of contraception from the time of the first administration of treatment or study medication until one month (30 days) following administration of the last treatment or dose of study medication. Acceptable methods include:

- A documented placement of an IUD or IUS and the use of a barrier method (condom or occlusive cap (diaphragm or cervical/vault caps) used with spermicidal foam/gel/film/cream/suppository)
- Documented tubal ligation (female sterilisation). In addition, a barrier method (condom or occlusive cap (diaphragm or cervical/vault caps) used with spermicidal foam/gel/film/cream/suppository) should also be used;
- Double barrier method: condom and occlusive cap (diaphragm or cervical/vault caps) with spermicidal foam/gel/film/cream/suppository
- Oral contraceptives (combination oestrogen/progesterone pills), injectable progesterone or subdermal implants and the use of a barrier method (condom or occlusive cap (diaphragm or cervical/vault caps) used with spermicidal foam/gel/film/cream/suppository)
- True abstinence when this is in line with the preferred and usual lifestyle of the subject. *Periodic abstinence (e.g., calendar, ovulation, symptothermal, post-ovulation methods) and withdrawal are not acceptable methods of contraception*

7.10. Day 28 post ictus (-6/+2 days):

The following procedures and assessments are to be carried out at the Day 28 post ictus visit:

- modified Rankin Score
- Short Form 36 Health Survey
- Subarachnoid Haemorrhage Outcome Tool
- Glasgow Outcome Scale (Extended)
- Concomitant Medication Review
- Protocol specific Safety Bloods and Urine (see Section 8.1)
- Blood sampling for HP & MDA, Proteomic /Genomic determination

Patients stop taking study medication at this visit – any remaining IMP will be collected.
It is anticipated that some patients will not be able to attend this visit in person. In this event, a member of the research team will visit the patient for sample collection (other assessments may be made by telephone).

7.11. Day 90 post ictus (± 14):

The following procedures and assessments are to be carried out at Day 90 post ictus:

- modified Rankin Score
- Short Form 36 Health Survey
- Checklist for Cognitive and Emotional Consequences
- Brain Injury Community Rehabilitation Outcomes Scale
- Subarachnoid Haemorrhage Outcome Tool
- Glasgow Outcome Scale (Extended)
- Concomitant Medication Review

A telephone interview with the patient or Personal Legal Representative will be arranged if attendance at the neurosurgical centre is not feasible. Where preferred by patients or Personal Legal Representatives questionnaires may be sent by mail or email.

7.12. Day 180 post ictus (± 28):

The following procedures and assessments are to be carried out at Day 180 post ictus:

- modified Rankin Score
- Short Form 36 Health Survey
- Checklist for Cognitive and Emotional Consequences
- Brain Injury Community Rehabilitation Outcomes Scale
- Subarachnoid Haemorrhage Outcome Tool
- Glasgow Outcome Scale (Extended)
- Concomitant Medication Review

- An MRI will be performed within 60 days of the Day 180 visit

A telephone interview with the patient or Personal Legal Representative will be arranged if attendance at the neurosurgical centre is not feasible. Where preferred by patients or Personal Legal Representatives questionnaires may be sent by mail or email.

7.13. Pharmacokinetic sub-study

A group of up to 12 patients, all of whom have been fitted with an EVD as part of normal treatment and prior to randomisation, may be selected for a pharmacokinetic sub-study. Additional patient consent will be required for the sub-study; in cases where patients lack capacity the consent of a Personal Legal Representative will be sought before any sub-study procedures are carried out.

The patients will, in addition to all other procedures (with the exception of lumbar puncture), undergo serial paired blood/CSF sampling (1 sample pre dose and hourly ± 5 minutes for 6 hours post dose) at one of the first three doses and day 7±1 post ictus.

7.14. Early Termination Visit

Patients have the right to withdraw from the trial at any time and for any reason. If a patient refuses to be seen for further visits the assessments at discharge & Day 28 should be performed at the time they have indicated that they will not attend for further visits, assuming they are available and consent to this.

7.15. Schedule of Assessments

	Pre-Dose Assessment	Dose	Post Dose	Ongoing assessments	Day 7 (±1)	At Discharge[1] (-2)[2]	Day 28 (-6/+2))	Day 90 (±14)	Day 180 (±28)
Time after Ictus →	0-48 hrs		12-24 hrs post dose						
Consent	X								
Inclusion/Exclusion	X								
Record results of CT/MRI & Fisher[3]	X								
Angiographic[4] assessment	X								
Medical history	X								
Physical exam	X								
Pregnancy Test[5]	X								
Con med review	X				X	X	X	X	X
Adverse events	X	X	X	X	X	X	X	X	X
Safety bloods[6]	X		X	X[7]	X	X	X[8]		
Lipid Profile & Coagulation Status[9]	X				X		X		
Safety urine[10]	X				X	X	X[8]		
Randomisation		X							
IMP Treatment[11]		X	X		X	X	X[8]		
TCD reading[12]	X[13]			X[14]	X				
Blood samples taken for HP, MDA & Proteomic / Genomic analysis (patients with an EVD fitted)[15]	X				X[16]		X[8]		
EVD CSF & paired blood sample taken for SFN & metabolites analysis (patients with an EVD fitted)[15]					X[16]				
Blood samples taken for HP, MDA & Proteomic / Genomic analysis (Patients without an EVD fitted)	X				X[16]		X[8]		
Lumbar Puncture CSF & paired blood samples for SFN & metabolites analysis (Patients without an EVD fitted)					X[16]				
Medication compliance			X		X	X	X		
WFNS (score required for randomisation to correct strata)	X								
mRS					X	X	X	X	X
SF36							X	X	X
CLCE-24								X	X
BICRO-39								X	X
SAHOT							X	X	X
GOSE							X	X	X
GCS				X		X			
MRI									X[17]

Main study: EVD Patients
Patients with an EVD fitted will also undergo paired single CSF/blood sampling on alternate days following the SAH (starting from day of EVD fitting) until day 14 or the EVD is removed – i.e. a single EVD sample is to be taken and paired with a single blood sample for determination of CSF/blood HP & MDA levels (and SFN at day 7).

[1]Discharge is when the consultant for the intervention of treating the SAH decides that their specialist care is no longer needed
[2]Procedures can be carried out up to 2 days prior to discharge
[3] Required for SAH diagnosis
[4] The Angiographic Assessment (CTA/DSA/MRA) and procedure planned and carried out (clipping/coiling) to be recorded – this can be carried out pre or post dose
[5] Urine or blood pregnancy test can be taken
[6] Safety bloods as per protocol requirements taken at predose, post dose, day 7, discharge and day 28 the tests required comprise:
Biochemistry: Sodium, Potassium, Urea, Creatinine, Glucose, Calcium, Total Bilirubin, Alkaline Phosphatase, Alanine Transaminase, Albumin, C-Reactive Protein /
Haematology: Haemoglobin, White Blood Cell Count, Neutrophils (Absolute), Lymphocytes (Absolute), Platelets
[7] Safety bloods carried out as part of normal SAH clinical care until no longer clinically indicated
[8] A member of the research team will visit the patient (other assessments may be made by telephone)
[9] Lipid Profile: LDL, HDL, Triglycerides, Total Cholesterol; coagulation status: INR or PT, APTR or APTT, & Fibrinogen (Clauss or Derived)
[10] Urine Microscopy. If no predose safety urine is available, then baseline urine taken prior to day 2 dosing will be acceptable
[11] Twice Daily approx. 12 hours apart until day 28 post ictus, time of dosing to be recorded. Dosing after discharge to be allowed dependent on 20 patient DSMB review.
[12] Reading Peak Velocity MCA flow
[13] Where possible - record timing of first TCD & whether pre- or post- dose
[14] TCD to be performed on alternate days (± 1) post ictus (starting day 3 (± 1) until at least Day 7 (± 1), or discharge whichever is sooner. Any additional TCD readings obtained after this point on clinical grounds will also be recorded.
[15]EVD Patients **do not** undergo Lumbar Puncture however if in the sub study set of patients, have serial paired blood and CSF samples as described in the protocol at one of the first 3 doses and on day 7.
[16]Paired blood sample taken at same time as Lumbar Puncture
[17]Within 60 days of 180 day target

Additional Interventions for Pharmacokinetic Sub-Study (Serial EVD sampling)

Substudy patients will undergo all procedures (except Lumbar Puncture) and *additionally* serial paired CSF_{EVD}/ blood sampling at one of the first three doses and on day 7 for determination of SFN metabolites:

	Pre-Dose Assessment	Dose	Post Dose	7 days (±1)	At Discharge (-2)	28 days (-6/+2)	90 days (±14)	180 days (±28)
Time after Ictus →	0-48 hrs							
Consent[18]	X							
Serial CSF Sampling (EVD)		X[19]		X[19]				
Serial blood SFN metabolites		X[20]		X[20]				

Note that alternate-day paired single CSF/blood sampling is also carried out with Sub-Study patients (as with all EVD patients): CSF sample for determination of HP & MDA levels & blood sample for HP, MDA & Proteomic/Genomic analysis.

[18] Substudy specific consent
[19] Trough EVD CSF sample taken prior to dose and every hour ± 5 minutes after dosing for six hours for determination of SFN metabolites
[20] Paired blood sample taken at same time as EVD sampling for determination of SFN metabolites

8. STUDY PROCEDURES / EVALUATIONS

8.1. Safety Measurements

The following safety parameters will be recorded according to the trial protocol:

- Concomitant medication
- Adverse events
- Escalation in grading of AE severity
- Safety blood tests
    - Safety Blood Tests should include the following parameters:
    - Biochemistry
        - Sodium
        - Potassium
        - Urea
        - Creatinine
        - Glucose
        - Calcium
        - Total bilirubin
        - Alkaline Phosphatase
        - Alanine Transaminase
        - Albumin
        - C-reactive protein
    - Haematology
        - Haemoglobin
        - White Blood Cell count
        - Neutrophils (absolute)
        - Lymphocytes (absolute)
        - Platelets
    - Lipid Profile
        - LDL
        - HDL
        - Triglycerides
        - Total Cholesterol
    - Coagulation Status
        - PT (or INR)
        - APTT (or APTR)
    - Fibrinogen

- Safety Urine tests (Microscopy)
    - Urine microscopy will be performed in accordance with local procedures. If no predose safety urine is available, then baseline urine taken prior to day 2 dosing will be acceptable.

8.2. Efficacy Measurements

The following efficacy parameters will be recorded according to the trial protocol:

- TCD
- WFNS
- mRS
- SF-36
- CLCE-24
- BICRO-39
- SAHOT
- GOSE
- GCS
- MRI

8.3. PK Measurements

The following PK parameters will be recorded according to the trial protocol:

Non-EVD Patients: i.e. patients will have a Lumbar Puncture for Collection of CSF

- Blood HP and MDA, Proteomic & Genomic concentration at baseline (pre dose 0-48 hours), D7 and D28
- Paired $CSF_{(Lumbar\ Puncture)}$/blood HP, MDA, Proteomic & Genomic & SFN/SFN metabolite concentration at Day 7

EVD Patients: i.e. Will not have a lumbar puncture

- Blood HP, MDA, Genomic & Proteomic concentration at baseline (pre dose 0-48 hours), D7 and D28
- Paired $CSF_{(EVD)}$/blood HP, MDA & Proteomic/Genomic concentration on alternate days (+/- 1 day) starting from day of EVD fitting to D14 or until EVD removal.
- Paired $CSF_{(EVD)}$/blood HP, MDA, Proteomic/Genomic & SFN/SFN metabolite concentration at day 7

Subset of 12 EVD Patients: In addition to all other all other sampling the following samples will be taken:

- Serial paired $CSF_{(EVD)}$/blood SFN/SFN metabolite concentration at one of the first 3 doses and at day 7

8.4. Definitions of Assessments

8.4.1. Fisher Grade

The Fisher grade is commonly used to predict the risk of cerebral vasospasm after SAH based on the amount of blood shown on initial CT scans within 5 days of SAH.

The Fisher grading system is split into four levels:

Grade 1: No blood

Grade 2: Diffuse or thin layer of blood less than 1 mm thick (interhemispheric, insular, or ambient cisterns)

Grade 3: Localized clots and/or layers of blood greater than 1 mm thick in the vertical plane

Grade 4: Intracerebral or intraventricular clots with diffuse or absent blood in basal cisterns

8.4.2. World Federation of Neurological Societies Grading System For Subarachnoid Hemorrhage (WFNS)

The WFNS is a 5 point scale that is a simple, reliable and clinically valid way to grade a patient with SAH. This system offers less inter-observer variability than some of the earlier classification systems. Randomisation of patients in this study is to either WFNS score strata 1-3 or WFNS score strata 4-5.

8.4.3. Modified Rankin Scale (mRS)

The mRS is widely used as a functional outcome measure in stroke. The purpose of the Rankin Focused Assessment (RFA) is to assign patients to mRS grades in a systematic way. The assessment consists of sections corresponding to levels of disability among stroke survivors on the mRS.

8.4.4. Short Form Health Survey (SF-36)

The SF-36 quality of life scale is a multi-purpose, short-form health survey with only 36 questions. It yields an 8-scale profile of functional health and well-being scores as well as psychometrically-based physical and mental health summary measures and a preference-based health utility index. It is a generic measure and the SF-36 has proven useful in surveys of general and specific populations, comparing the relative burden of diseases, and in differentiating the health benefits produced by a wide range of different treatments.

8.4.5. Checklist for Cognitive and Emotional Consequences (CLCE-24)

The CLCE-24 is a checklist used for identification of cognitive and emotional problems after stroke. The CLCE-24 is a usable and valid instrument for cognitive screening by health care professionals in the stroke service in the chronic phase after stroke.

8.4.6. Brain Injury Community Rehabilitation Outcomes Scale (BICRO-39)

The BICRO-39 questionnaire is a multidimensional, quantitative assessment designed to measure community functioning in areas of activity, social participation, and psychological components. This assessment requires patients and/or caregivers to evaluate the level of functioning on each item pre- and post-injury. It can also be used to track changes in performance across time. Functional areas assessed include personal care, psychological, socializing, self-organization, mobility, productive employment, and family contact.

The questionnaire consists of three forms: patient pre-injury (P-PRE), patient post-injury (P-POST) and carer post-injury (C-POST) with each item on the questionnaires assigned a score of zero to five.

8.4.7. Subarachnoid Haemorrhage Outcome Tool (SAHOT)

The SAHOT is a new SAH-specific outcome assessment form that is filled by patient and Personal Legal Representative. SAHOT was designed and developed in Southampton University to assess recovery following SAH. It consists of a series of questions which aim to assess the degree of change in the above fields following SAH. The degree of change is graded into five main categories. This questionnaire demonstrates the impact of SAH in four main aspects of daily life including: general aspects, physical, cognitive and behavioural/psychological.

8.4.8. Glasgow Outcome Scale Extended (GOSE)

The Glasgow Outcome Scale is a global scale for functional outcome that rates patient status into one of five categories: Dead, Vegetative State, Severe Disability, Moderate Disability or Good Recovery. The Extended Glasgow Outcome Scale (GOSE) provides more detailed categorization into eight categories by subdividing the categories of severe disability, moderate disability and good recovery into a lower and upper category.

A structured interviews guide will be used for this trial.

8.4.9. Glasgow Coma Score (GCS)

The GCS is a standard measure to assess the level of consciousness of patients who have sustained head injuries. The GCS is part of standard treatment protocols and used for general decision-making for critically ill patients. It is an objective and reliable scale employed for initial and subsequent assessments. It consists of three items, Eyes (E), Verbal (V), and Motor (M) with each domain scoring a minimum of 1 giving an overall score ranging from 3 to 15.

8.5. Clinical Evaluations

8.5.1. Medical History

All patients will typically have full medical history taken followed by thorough physical examination as part of their admission routine.

8.5.2. Radiology

CT (or MRI) will be used to confirm the presence of SAH.

CTA, DSA or MRA will be used to confirm the presence of an aneurysm in this cohort of patients as per normal clinical care.

8.5.3. TCD Recordings

Trans Cranial Doppler readings are obtained on alternate days (± 1) post ictus (starting day 3 (± 1) until at least Day 7 (± 1)) or discharge whichever is sooner. Any additional TCD readings obtained after this point will also be recorded as this may continue as per usual until the subject is discharged from the neurosurgical Centre. TCDs will be performed by an experienced member of the medical physics or neuro-intensive care team or other appropriately trained personnel who have otherwise carried out the same procedure on the same group of patients. The readings will be kept in the patient's medical notes and values will be entered on to the assessment eCRF by the research team.

8.5.4. MRI

All patients will have an MRI with Susceptibility Weighted Imaging (SWI) sequences performed at six months (+/- 60 days).

In those patients whose aneurysm have been coiled (expected to be ~70% of patients) it would be expected that if they had recovered sufficiently that they would undergo an MRI on clinical grounds at this time. For these patients the SWI sequences will be added to the same MRI session as their scheduled clinical scan. For patients whose aneurysms have been surgically clipped or patients who have been coiled but are elderly in whom retreatment of any aneurysmal recurrence would not normally be contemplated, this is likely to represent an extra study intervention.

To quantify brain atrophy, 3D T1-weighted images (with high resolution isotropic voxels) will be acquired using neurosurgical centre's standard protocol. Whole brain atrophy will be assessed by using the modified cella media index and by more detailed analysis deriving CSF to intracranial volume ratio after image segmentation (using Statistical Parametric Mapping Software). Voxel-based morphometry will be used to identify and quantify regional areas of atrophy. Clip/coil artefact will be masked to ensure correct normalization of images and segmentation into CSF, grey matter and white matter.

To quantify iron, fully flow-compensated, 3D, high-resolution, gradient-echo Susceptibility-Weighted Imaging sequences will be used to collect magnitude and phase data. The phase images will be processed and analysed (using Signal Processing in NMR software) to quantify iron in regions of interest, such as orbitofrontal cortex, inferior temporal gyrus, hippocampus, parahippocampal gyrus and thalamus – i.e. the basal regions likely to be most exposed to haemoglobin following SAH.

8.6. Clinical Laboratory Evaluations

The following investigations will be performed during the acute admission of the subject to the neurosurgical centre. The details will be recorded on the eCRF form and upon completion they will be filed in the Trial Master File (TMF). Samples will be analysed at the local laboratory at the neurosurgical centre.

8.6.1. Blood Sampling

During the inpatient stay at the neurosurgical centre, routine blood tests including FBC, U&Es, LFTs and CRP are taken regularly, initially daily and later on an alternate daily basis as part of their care in NICU or neurosurgical wards. The results from these tests will be required for study purposes and at several specific time points. These will be measured at baseline on admission (pre-dose assessment), within 24 hours of first dose, at day 7 post ictus, discharge and at day 28.

As well as routine blood tests Coagulation status (INR or PT, APTR or APTT, & Fibrinogen (Clauss or Derived)) will be measured at Pre-Dose Assessment, day 7 and day 28.

8.6.2. Pregnancy Testing

Women of childbearing potential (all premenopausal women, or in cases where menstrual status cannot be ascertained including women under the age of 55) should have a urine pregnancy test performed at screening before study drug initiation.

8.6.3. Lumbar Puncture

Patients (without an External Ventricular Drain fitted) will have a lumbar puncture performed on day 7 following the SAH for study purposes. In many patients they will have this performed for clinical reasons during this window and thus the test will not be repeated. Around 30% of eligible patients will have an External Ventricular Drain (EVD) sited for clinical reasons and CSF will be obtained through the EVD i.e. if patients have an EVD fitted lumbar puncture is not carried out.

For the remaining patients that are required to have a lumbar puncture for study purposes alone this can be justified by:

1 the known high incidence of low grade hydrocephalus which may be diagnosed and relieved by lumbar puncture

2 the known advantage of CSF drainage to reduce blood load and reduce inflammation

The benefits of these effects have been demonstrated previously in a randomised controlled trial [9] showing a significantly better short term outcome with CSF drainage although the significance of this effect was lost on longer term follow up; hence it has not necessarily been seen as cost effective and universally adopted. However, in the context of this study there is good evidence that CSF drainage is at least beneficial in the short term and not harmful in the long term and can thus be justified as a study specific intervention.

Opening pressure will be recorded and approximately 17 ml of CSF will be taken at the time of lumbar puncture for research purposes or as much as is required to halve the pressure (as per routine clinical practice). Of the volume collected, 1 ml will be sent for routine microbiological assessment (microscopy culture and sensitivity). The remainder will be taken for study purposes to measure HP, MDA and SFN levels. Paired blood samples will be taken at the same time (for HP, MDA & SFN determination).

In the event that patients undergo further lumbar puncture outside the day 7 window for clinical reasons, CSF not utilised for clinical purposes and that would otherwise be discarded may be retained for future research purposes. The results from these samples will not be included in the study report.

8.6.4. EVD Sampling

Around 30% of eligible patients will have an External Ventricular Drain (EVD) sited for clinical reasons. This cohort would normally, on clinical grounds, have daily blood samples and regular CSF sampling ranging from daily to twice weekly. In this study, for this cohort of patients approximately 10ml CSF will be taken on alternate days for study purposes (starting on day of EVD fitting) until day 14 or the EVD is removed. Paired blood samples will be taken with every EVD CSF sample. The samples will be used to determine CSF/blood HP & MDA levels (and CSF/blood SFN levels at day 7 requiring a further 10 ml CSF).

Twelve patients may participate in a Pharmacokinetic sub-study, all of whom have been fitted with an EVD as part of normal treatment and prior to randomisation.
The patients will, in addition to all other procedures (with the exception of lumbar puncture), undergo serial CSF sampling (1 sample pre dose and hourly ± 5 minutes for 6 hours post dose) at one of the first three doses and day 7±1 post ictus..

In the case of Serial CSF_{EVD} sampling, approximately 5ml CSF will be removed per time point. The samples will be used to determine CSF SFN levels.

8.6.5. Non-Standard Assays or Procedures

Blood samples (up to a maximum of 25ml in one sample) will be taken for the following purposes:

- Determination of SFN/SFN metabolite, HP and MDA levels

- Proteomic/genomic Evaluation
    - DNA and RNA will be extracted and used for subgroup analysis and evaluation of the effects of SFX-01. These evaluations are for research purposes and may be stored for future genetic testing; the results will not be included in the study report.

9. ADVERSE EVENT REPORTING

All AEs will be reported from the time a signed informed consent form is obtained until 30 days after the last dose of study medication, Adverse events occurring after 30 days following the last dose of study medication must also be reported if considered related to study drug.

9.1. Definitions

Adverse Event:
Any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment.

An adverse event can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom or disease temporally associated with the use a medicinal (investigational) product, whether or not related to the medicinal (investigational) product.

Adverse Drug Reaction:
Untoward and unintended responses to an investigational medicinal product related to any dose administered.

All adverse events judged by either the reporting Investigator or the Sponsor as having a reasonable causal relationship to a medicinal product qualify are adverse reactions. The expression “reasonable causal relationship” means to convey in general that there are facts or evidence meant to suggest a causal relationship.

Serious Adverse Event (SAE):
Any untoward medical occurrence or effect that at any dose falls in one or more of the following categories:

- Results in death
- Is life-threatening
- Requires hospitalisation or prolongation of existing inpatients’ hospitalisation. Hospitalisation refers to a situation whereby an AE is associated with unplanned overnight admission into hospital.
- Results in persistent or significant disability or incapacity
- Is a congenital anomaly or birth defect
- Is a medically significant adverse event
- adverse events of special interest

9.2. Expectedness

An expected adverse reaction is an adverse reaction, the nature or severity of which is consistent with the applicable product information in the Investigator’s Brochure, otherwise it is considered unexpected.

9.3. Intensity of Adverse Event

Each adverse event must be rated according to the following:

Mild: A type of adverse event that is usually transient and may require only minimal treatment or therapeutic intervention. The event does not generally interfere with usual activities of daily living.

Moderate: A type of adverse event that is usually alleviated with additional specific therapeutic intervention. The event interferes with usual activities of daily living, causing discomfort, but poses no significant or permanent risk of harm to the subject.

Severe: Marked limitation in activity; medical intervention/therapy required, hospitalisation possible

Life-threatening: Extreme limitation in activity, significant medical intervention/therapy required; hospitalisation care possible.

9.4. Causality Assessment

Certain	A clinical event, including laboratory test abnormality, occurring in a plausible time relationship to drug administration, and which cannot be explained by concurrent disease or other drugs or chemicals. The response to withdrawal of the drug (dechallenge) should be clinically plausible. The event must be definitive pharmacologically or phenomenologically, using a satisfactory rechallenge procedure if necessary.
Probable / likely	A clinical event, including laboratory test abnormality, with a reasonable time sequence to administration of the drug, unlikely to be attributed to concurrent disease or other drugs or chemicals, and which follows a clinically reasonable response on withdrawal (dechallenge). Rechallenge information is not required to fulfil this definition.
Possible	A clinical event, including laboratory test abnormality, with a reasonable time sequence to administration of the drug, but which could also be explained by concurrent disease or other drugs or chemicals. Information on drug withdrawal may be lacking or unclear.
Unlikely	A clinical event, including laboratory test abnormality with a temporal relationship to drug administration which makes a causal relationship improbable, and in which other drugs, chemicals or underlying disease provide plausible explanations.
Conditional / unclassified	A clinical event, including laboratory test abnormality, reported as an adverse reaction, about which more data is essential for a proper assessment or the additional data are under examination.

Unassessible / unclassifiable	A report suggesting an adverse reaction which cannot be judged because information is insufficient or contradictory, and which cannot be supplemented.

9.5. Action Taken Regarding the Study Drug

The action taken regarding study drug must be described by selecting one of the following:

- Permanently discontinued
- Stopped temporarily
- Dose reduced
- Dose increased
- No action taken
- Unknown / not applicable

9.6. Outcome

Each AE must be rated by selecting one of the following:

- Recovered / resolved
- Recovering / resolving
- Not recovered / not resolved
- Recovered with sequelae / resolved with sequelae
- Fatal
- Unknown

9.7. Recording Adverse Events

It is the responsibility of the investigator to collect all AEs (both serious and non-serious).

All AEs occurring during the study must be documented on the appropriate section of the case report form (CRF).

If AE is considered serious, it must also be recorded on the Serious Adverse Event Form provided separately.

9.8. Adverse Reaction to SFX-01

Any non-serious adverse events that are deemed to be directly related to the IMP should be reported within 24 hours of awareness.

All Adverse Reactions must be emailed or faxed to Diamond Pharma Services:
Email: PVServices@diamondpharmaservices.com
Fax number: +44 (0)1279 418 964

9.9. Serious Adverse Events

All SAEs must be reported to the CI, CRO and sponsor within 24 hours of awareness, regardless of causal relationship. An SAE form must be filled in by a member of the research team and kept in the TMF.

All SAEs occurring until the end of the trial must be reported by fax immediately by the Investigator or designated assistant is made aware of the event and by full report as soon as possible thereafter.

All SAEs must be emailed or faxed to Diamond Pharma Services Ltd:
Email: PVServices@diamondpharmaservices.com
Fax number: +44 (0)1279 418 964

Where the investigator requires advice regarding the handling of Serious Adverse Events, the contact in case of emergency is:

Diamond Pharma Services Ltd Emergency 24 hour phone number: +44 (0) 1249 406 759

Pregnancies occurring during the study must be reported immediately by fax using the SAE Form.

Diamond Pharma Services will report any SUSARs occurring in the trial to the relevant CA. Evgen Pharma plc will report SUSARs to relevant Research Ethics Committees (REC(s)) and the DSMB as outlined in section 25.

Evgen Pharma plc and Diamond Pharma Services Ltd will keep the Investigator and DSMB (section 25) informed of all SAEs reported to them for the product under investigation, from anywhere in the world, for the duration of the trial at a frequency appropriate to the trial.

In addition, any new safety information that would adversely affect the safety of patients or the conduct of the trial will be reported by Evgen Pharma plc to the CAs, RECs, DSMB and Investigators. If the trial is to be suspended as a result of a SUSAR, or due to any urgent safety measure taken, the CA and REC will be notified as soon as possible and within three days of the decision.

Evgen Pharma plc will submit Safety Reports to the CAs and RECs annually or more frequently if so requested.

10. DATA MANAGEMENT

Data will be recorded on an eCRF by the Investigator (or designee). The database, data entry and electronic checks will be developed using a Clinical Database Management System. Computerised data cleaning checks will be used in addition to manual review to check for discrepancies and to ensure consistency and completeness of the data. An electronic audit trail system will be used to track all data changes in the database.

Data Clarification Forms (DCFs) will be generated in order to clarify any issues which arise during data cleaning. These will be distributed to site for resolving and sign off by the Investigator.

A 100 % quality control check of the data entry will be performed on a randomly selected sample of the eCRFs.

Medical history findings and adverse events will be coded using the MedDRA dictionary; medications will be coded using the World Health Organisation Drug dictionary.

10.1. Trial Documentation and Trial Confidentiality

10.1.1. Trial Documentation, eCRFs and Document Keeping

The Investigator must generate and maintain adequate records (patient medical records, Case Report Forms, source documents) to enable the conduct of this trial to be fully documented. Each patient enrolled into the trial must have an eCRF completed and the eCRF must be reviewed and electronically signed off by the investigator. This applies to those patients who failed to complete the trial (even during the pre-randomisation period). eCRFs are to be completed either at the time of the patient's visit or as soon as possible after the visit so that they always reflect the latest observations on the patients participating in the study. The investigator must electronically verify that all data entries in the eCRFs are accurate and correct. The nature and location of all source documents will be identified to ensure that all sources of original data required to complete the eCRF are known to the company and investigational staff are accessible for verification by the clinical monitor. If electronic records are maintained, the method of verification must be discussed with the investigational staff. A source data verification log will be prepared by TCTC. This will describe the proportion of eCRF data that will be verified by the monitor against the patients' medical records and source data.

The sponsor recommends that the author of an entry in the source documents be identifiable. Direct access to source documentation (medical records) must be allowed for the purpose of verifying that the data recorded on the eCRF are consistent with the original source data.

If data are recorded directly into the eCRF, there should be, at a minimum, an entry in the medical record that each of the assessments was performed; who performed it and the date it was done.

The eCRF will be compared with the source documents to ensure that there are no discrepancies between critical data. All entries, corrections, and alterations are to be made by the responsible investigator or an authorised member of the investigational staff.

Data clarification and query resolution will be conducted on an ongoing basis by the monitor and the contract data management company. Sponsor will have overall responsibility for the data.

The Principal Investigator must be aware of their responsibility to retain patient identification codes in line with regulatory requirements after completion or discontinuation of the trial. If a patient ceases treatment prematurely, then the reason must be noted in the eCRF. If a patient ceases treatment because of an adverse event, reasonable efforts must be made to clearly document the outcome.

The Principal Investigator will allow authorised Sponsor personnel, auditors and regulatory authorities direct access to the patients' medical records.

Copies of protocols, eCRF page/printouts, originals of test results, reports, drug dispensing logs, correspondence, records of informed consent or other documents pertaining to the conduct of the trial must be kept on file by the Investigator in line with regulatory requirements or for the period of time specified by local law for the preservation of hospital patient documents, whichever is the longer. No trial documents should be destroyed without prior written agreement between Sponsor and the Investigator. Where storage at the centre is limited,

the Sponsor may make arrangement for documents to be stored at an independent data archiving facility on behalf of the Principal Investigator. Should the Investigator wish to assign the trial records to another party, or move them to another location, the clinical trial monitor must be consulted.

10.1.2. Confidentiality of Trial Documents and Patient Records

The Investigator must ensure the patients' anonymity is maintained. On CRFs or other documents submitted to TCTC/the Sponsor/third party contractor, patients must NOT be identified by their names, but by an identification code (usually their trial number). The Investigator will be responsible for maintaining a separate log of patients' codes, names and unique identifiers. This log will be maintained as required by applicable regulatory requirements. Documents not for submission to TCTC /the Sponsor/third party contractor, e.g. patients' written consent forms, must be maintained by the Investigator in strict confidence.

11. STATISTICAL ANALYSIS PLAN

A detailed statistical analysis plan (SAP) will be produced after having finalised the protocol and prior to database lock.

11.1. Sample Size

Up to 120 patients may be recruited and enrolled into the trial in order to provide 90 who will meet the per protocol criteria and be analysed for the efficacy analyses.

No formal sample size calculation has been carried out; the Power associated with a sample size of 90 is based on the following assumptions:

- The error probability for the Type-I error should not exceed 5% for a 1-sided test;
- The primary endpoint will be compared between treatment groups by means of a t-test
- The mean maximum MCA flow velocity for patients treated with SFX-01 is estimated as 175 cm/s [31] and
- The standard deviation is set to 50 cm/s

Under these assumptions 90 patients will give 80% power to detect a difference in maximum MCA velocity which is approximately half of the standard deviation of the mean value. The standard deviation was assumed to be approximately 30% of the mean value.

11.1 Populations for analysis

The following populations will be considered for the analysis:

- **Intention-to-Treat population (ITT):** all randomised patients who receive at least one dose of study medication and with any post-dose efficacy evaluations.
  Patients where the time from ictus to admission is unknown are to be considered as part of the ITT population
- **Per-protocol population (PPP):** The Per Protocol Population (for Primary analysis) will be considered to be those patients in the ITT population that have been dosed for a minimum to day 7 post ictus without any major protocol violations (e.g., wrong inclusions, forbidden concomitant medications, etc.). Exact definition of major protocol deviations will be discussed by the clinical team case by case during the Blind Review of the data and described in the Blind Review document. Protocol violations will be considered for each treatment period separately.

- **Safety population:** all randomised patients who have taken at least one dose of study medication.

11.2. Statistical parameters and tests

11.2.1. Primary outcome

Safety

- Concomitant medication
- Adverse events
- Escalation in grading of AE severity
- FBC, U&Es, LFT, CRP & Urine Microscopy
- INR or PT, APTR or APTT, & Fibrinogen (Clauss or Derived) at 7 & 28 days

Pharmacokinetic

- Presence of SFN in CSF

Efficacy

- The maximum MCA flow velocity determined using TCD.
  Treatment groups will be compared using a t-test.

11.2.2. Secondary outcomes

- modified Rankin Scale (mRS), at 7 days, discharge, 28, 90 and 180 days.
  Treatment groups will be compared using a van Elteren test.
- Incidence of Delayed Cerebral Ischaemia (DCI) defined as a new focal deficit or reduction in (Glasgow Coma Scale) GCS ≥2 if not explained by other causes (i.e re-bleed, hydrocephalus, seizure, meningitis, sepsis or hyponatraemia)
  Treatment groups will be compared using a chi-square test.
- Incidence of new cerebral infarct on Computed Tomography (CT) or Magnetic Resonance Imaging (MRI)
  Treatment groups will be compared using a chi-square test.
- Institution of hypertensive (triple H) therapy for presumed DCI
  Treatment groups will be compared using a chi-square test.
- SF-36 quality of life survey at 28, 90 & 180 days.
  Treatment groups will be compared using a t-test.
- Checklist for Cognitive and Emotional Consequences (CLCE-24), Brain Injury Community Rehabilitation Outcomes Scale (BICRO-39), 90 & 180 days.
  Treatment groups will be compared using a van Elteren test.
- Subarachnoid Haemorrhage Outcome Tool (SAHOT) and Glasgow Outcome Scale – Extended (GOSE) at 28, 90 & 180 days.
  Treatment groups will be compared using a van Elteren test.
- Length of acute hospital stay
  Treatment groups will be compared using a Wilcoxon-Mann-Whitney-test.
- Discharge location
  Treatment groups will be compared using a chi-square test.

- Amount of iron identified on MRI Susceptibility Weighted Imaging (SWI) 180 days after start of treatment.
  Treatment groups will be compared using a t-test.
- Cortical atrophy on T1 MRI at 180 days after start of treatment
  Voxel-based morphometry will be used to identify and quantify regional areas of atrophy

Non-EVD Patients: i.e. patients will have a Lumbar Puncture for collection of CSF

- Blood HP and MDA, Proteomic & Genomic concentration at baseline (pre dose 0-48 hours), D7 and D28
- Paired $CSF_{(Lumbar\ Puncture)}$/blood HP, MDA, Proteomic & Genomic & SFN/SFN metabolite concentration at Day 7

EVD Patients: (i.e. will not have a lumbar puncture)

- Blood HP and MDA, Proteomic & Genomic concentration at baseline (pre dose 0-48 hours), D7 and D28
- Paired $CSF_{(EVD)}$/blood HP & MDA, Proteomic & Genomic concentration on alternate days (+/- 1 day) starting on day of EVD fitting until D14 or the EVD removal
- Paired $CSF_{(EVD)}$/blood HP, MDA, Proteomic /Genomic & SFN/SFN metabolite concentration at day 7

Subset of 12 EVD Patients: In addition to all other sampling the following samples will be taken:

- Serial paired $CSF_{(EVD)}$/blood SFN/SFN metabolite concentration at one of the first 3 doses and day 7

Measured PK-variables will be log-transformed, if necessary, and descriptively displayed using Box-plots.

12. ETHICS COMMITTEE / IRB APPROVAL

The study proposal will be submitted to the Ethics Committee in accordance with the national requirements.

The EC shall give its opinion in writing before the clinical trial commences. The investigator should provide written reports to the EC annually or more frequently if requested on any changes significantly affecting the conduct of the trial and / or increasing risk to the subjects.

13. REGULATORY REQUIREMENTS

The study will be authorised by the Medicines and Healthcare products Regulatory Agency (MHRA) in the UK.

Enrolment of subjects will not start until approval has been received from both the Ethics Committee(s) and Competent authorities.

The study will be conducted in accordance with the Declaration of Helsinki, Good Clinical Practice (ICH-GCP) and all other national requirements.

14. INFORMED CONSENT

Due to the nature of SAH it is expected that the majority of participants will be considered to be 'incapacitated adults' at the time of entry into the study where an incapacitated adult is defined as "an adult unable by virtue of physical or mental incapacity to give informed consent".

The hierarchy for consent is considered to be:

1. Patients with Capacity Those patients able to give written informed consent
2. Personal Legal Representative A person not connected with the conduct of the trial who is: (a) Suitable to act as the legal representative by virtue of their relationship with the adult, <u>and</u> (b) Available and willing to do so
3. Professional legal representative A person not connected with the conduct of the trial who is: (a) The doctor primarily responsible for the adult's medical treatment, or (b) A person nominated by the relevant health care provider A professional legal representative may be approached if no suitable personal legal representative is available
4. No representative In emergency situations where the treatment to be given to an incapacitated adult as part of the trial needs to be given urgently, time may not allow for the written consent of a legal representative to be obtained first. NOTE: Option 4. No representative - Dosing is *only* permissible where local regulations allow

<u>Patients with capacity</u>

Written and verbal versions of the patient information and informed consent form will be presented to the participants detailing the exact nature of the trial, what it will involve for the subject, the implications and constraints of the protocol and the known side effects and risks involved in taking part. It will clearly state that the participant is free to withdraw from the trial at any time for any reason without prejudice to future care and with no obligation to give the reason for withdrawal.

The patients will be allowed as much time as they need in which to decide whether to participate in the trial. The 48 hour timeline for trial medication initiation will not be used to put pressure on the patient to make a decision.

The Investigator should explain to the patient that they are at liberty to refuse entry to the trial or, should they decide to participate, to withdraw from the trial at any time. Such a decision will not, in any way, impinge on their future management.

Written Informed Consent will be obtained by means of participant dated signature with dated signature of the person who presented and obtained the Informed Consent. The person who obtains the consent must be suitably qualified and experienced and have been authorised to do so by the Chief/Principal Investigator. A copy of the signed Informed Consent will be given to the participant and copy will be kept in the patient's notes. The original signed form will be retained in the trial site file (TSF).

Patients lacking capacity with Personal Legal Representative immediately available
Where patients lack capacity and the Personal Legal Representative is immediately available in person an identical approach will be taken substituting the Personal Legal Representative for the patient.

Recording consent of the Personal Legal Representative by telephone is not permitted.

For patients that were unconscious at screening, and informed consent was obtained from their Personal Legal Representative, written informed consent will be obtained and documented from the patient as soon as they regain consciousness sufficiently to do so, respecting their right to withdraw from the study should they wish to.

If the patient has not regained capacity by the time of their 6 month follow up no further attempts will be made to obtain consent.

Patients lacking capacity where Personal Legal Representative is not immediately available
For patients lacking capacity where a Personal Representative is not immediately available in person, a Professional Legal Representative will be sought (providing they are not part of the study team). If they are in attendance in person they will discuss the trial with the research team and complete a consent form if they feel it is appropriate for the subject to participate in the trial.

If they are not in attendance in person they will be contacted by telephone and their opinion sought. If in agreement, the study team will document the verbal consent in the patient notes (details of the representative, date and time of the telephone call, summary of the discussion and Informed Consent process and version of Informed Consent Form), the patient will be enrolled and the Professional Legal Representative will complete a consent form the next time they attend the patient.

For those patients that were unconscious at screening, with informed consent obtained from their Professional Legal Representative, written informed consent will be obtained and documented from the patient as soon as they regain consciousness sufficiently to do so.

For patients lacking capacity and where a Personal Legal Representative was not immediately available, written informed consent will be obtained and documented from the Personal Legal Representative at the earliest opportunity, respecting their right to withdraw the patient from the study should they wish to.

If the patient has not regained capacity by the time of their 6 month follow up no further attempts will be made to obtain consent.

Patients lacking capacity where Professional Legal Representative is not immediately available
For patients lacking capacity where a Personal Representative or Professional Legal Representative is not immediately available (including Professional Legal Representative unavailable by telephone), the study team will discuss potential recruitment of the patient into the study and complete a consent form if they feel it is appropriate for the subject to participate in the trial. The patient will be randomised and receive the first two doses whilst consent is being sought.

Note that this is only permissible where local regulations allow; if local regulations do not allow emergency dosing without consent the patient shall not be enrolled into the study.

In the instance where a patient has been entered into the trial prior to informed consent being obtained (i.e. through the emergency consent procedure) and consent is subsequently refused or not obtained within 24 hours by the patient and/or legal representative, the participant shall be withdrawn and replaced.

The consent of the patient, Personal Legal Representative and Professional Legal Representative will continue to be sought; if consent has not been obtained when the third dose is due the patient shall be withdrawn from the study.

EVD Sub-study
Only Patient consent or that of a Personal Legal Representative (in the case of patients lacking capacity) will be sought prior to any sub-study procedures being carried out.

15. DIRECT ACCESS TO SOURCE DOCUMENTATION / DATA

The investigator must permit trial-related monitoring, audits, Ethics Committee review or regulatory inspection, providing direct access to source data / documents.

16. STUDY MONITORING

It is understood that the study monitor(s) will contact and visit the investigator/clinical site before the study, regularly throughout the study and after the study has been completed. At these visits the monitor(s) will inspect various study records; case report forms, investigator site file and source data, provided that subject confidentiality is respected. The investigator and / or site staff will be expected to be available if requested by the monitor.

17. QUALITY ASSURANCE

The sponsor Evgen Pharma plc may perform an audit at any time according to the sponsor's Standard Operating Procedure, in order to verify whether the study is being conducted according to GCP.

18. TRIAL SCHEDULE

This trial is expected to start in Quarter 2 2016, with all patients recruited and the treatment phase completed Quarter 2 2018. The integrated clinical and statistical report will be completed Quarter 4 2018.

19. INSURANCE

Appropriate insurance cover has been undertaken in favour of patients participating in clinical trials. The cover is provided to the patient on terms and conditions of the clinical trial insurance. Insurance cover exists for health damages as a result of measures carried out in connection with the clinical trial.

20. CONFIDENTIALITY

All study documents are provided by the sponsor in confidence to the investigator and appointed staff. No study material may be disclosed to any party not directly involved in the study without written permission from the sponsor.

The investigator must assure that subject's anonymity will be provided. The investigator will keep a separate list with at least the initials, the subject's study number, names, addresses and telephone numbers. The investigator will maintain this for as long as requested by the sponsor.

21. DATA PROTECTION

Details of access to the patients' data, conforming to the requirements of EU Directive 95/46/EC, will be fully described within the patient information sheet. The consequence of the patients' withdrawal of consent with regards to the use of data will also be described.

22. PREMATURE TERMINATION OF THE STUDY

Both the sponsor and the investigator reserve the right to terminate the study at any time. Should this be necessary, the procedures for an early termination or temporary halt will be arranged after consultation with all parties.

23. RECORD RETENTION

After completion of the study, all documents and data relating to the study will be kept in an orderly manner by the Investigator in a secure study file.

Essential documents must be retained for at least two years after the final marketing approval in an ICH region or until two years have elapsed since the formal interruption of the clinical development of the product under study.

It is the responsibility of the sponsor to inform the investigator of when these documents can be destroyed. The Investigator must contact the sponsor before destroying any trial-related documentation. In addition, all subjects' medical records and other source documentation will be kept for the maximum time permitted by the institution.

24. PUBLICATION OF RESULTS

The sponsor is entitled to publish and/or present any results at scientific meetings, and to submit clinical trial data to national and international Regulatory Authorities. The sponsor reserves the right to use such data for industrial purposes.

Investigators must inform the sponsor before using the results of the study for publication or presentation and agree to provide the sponsor with a copy of the proposed presentation.

25. DATA SAFETY MONITORING BOARD (DSMB)

A committee will be set-up to monitor safety throughout the trial period. The committee will comprise a group of independent experts and will include a chairperson, a specialist neurosurgeon (experienced in SAH) and a statistician all of whom will be independent of the sponsor and will not be involved in the conduct of the trial.

A charter describing how the DSMB works and how it communicates with other study participants (e.g. steering committee) will be prepared.

The DSMB will review unblinded study information which will include:

- List of any protocol violations
- Numbers of patient withdrawals/reason for withdrawal
- Adverse/serious adverse events
- Laboratory data

Following review of the safety data, the committee will prepare written reports (quarterly at a minimum) which will be forwarded to the steering committee advising of any recommendations regarding modifications, continuation or termination of the study.

Where changes in the study conduct are recommended to the steering committee, sufficient (blinded) information will be provided to allow the sponsor to decide whether and how to implement these recommendations.

DSMB Meetings:

The DSMB will convene after 20 patients have been dosed to day 7 post ictus (with adequate safety assessment data) as in-patients in tertiary care for a formal safety review.
The safety review shall make a decision on the acceptability of discharging patients from tertiary care with SFX-01 to complete the dosing course to day 28.
The assessment will provide four possible outcomes:

1. Proceed as planned including allowing continuation of dosing as outpatients from tertiary care
2. Continue with tertiary care inpatient dosing only
3. Proceed after substantial modification of the protocol
4. Discontinue the study

Recruitment will continue throughout the 20 patient DSMB – note that all patients (including those discharged home) complete all the safety assessments.

Data Safety Monitoring Board

The DSMB will convene under the following circumstances:

- The DSMB must meet once the 20th patient has been dosed to day 7 post ictus
- The DSMB must meet as soon as there has been a SUSAR
- The DSMB must meet if 2 patients have a grading change in AE severity (from mild/ moderate to severe or life threatening)
- The DSMB can meet at any point deemed necessary

Study Stopping Rules

The clinical investigation can be placed on hold / stopped early for two reasons and will be based on clinical judgement:

- The DSMB will consider recommending that the study is placed on hold or stopped if the adverse events associated with participation in the study are considered unacceptable.

- The DSMB will consider recommending that the study is placed on hold or stopped if the adverse events associated with SFX-01, in their opinion, significantly outnumber (in frequency or intensity) the adverse events associated with the normal standard of care.

26. STEERING COMMITTEE

The steering committee for the SFX-01 clinical programme will be called the SFX-01 Executive Committee. The executive committee (who will be blinded) will comprise at a minimum, representative(s) of the sponsor (Chief Medical Officer) and the Chief Investigator; the committee will receive and review the reports from the DSMB, and take action as appropriate. This may be a decision to either modify, continue or terminate the study.

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28. APPENDICES

28.1. Appendix 1 – Declaration Of Helsinki - 1996

Recommendations Guiding Medical Physicians in Biomedical Research Involving Human Volunteers

Adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964, amended by the 29th World Medical Assembly, Tokyo, Japan, October 1975 and the 35th World Medical Assembly, Venice, Italy, October 1983 and revised 41st World Medical Assembly Hong Kong, 1989 and by the 48th World Medical Assembly, South Africa, October 1996

Introduction

It is the mission of the physician to safeguard the health of the people. His or her knowledge and conscience are dedicated to the fulfilment of this mission.

The Declaration of Geneva of the World Medical Association binds the physician with the words, "The health of my patient will be my first consideration", and the International Code of Medical Ethics declares that, "A physician shall act only in the patient's interest when providing medical care which might have the effect of weakening the physical and mental condition of the patient".

The purpose of biomedical research involving human volunteers must be to improve diagnostic, therapeutic and prophylactic procedures and the understanding of the aetiology and pathogenesis of disease.

In current medical practice most diagnostic, therapeutic or prophylactic procedures involve hazards. This applies especially to biomedical research.

Medical progress is based on research which ultimately must rest in part on experimentation involving human volunteers.

In the field of biomedical research a fundamental distinction must be recognised between medical research in which the aim is essentially diagnostic or therapeutic for a patient, and medical research, the essential object of which is purely scientific and without implying direct diagnostic or therapeutic value to the person volunteered to the research.

Special caution must be exercised in the conduct of research which may affect the environment, and the welfare of animals used for research must be respected.

Because it is essential that the results of laboratory experiments be applied to human beings to further scientific knowledge and to help suffering humanity, the World Medical Association has prepared the following recommendations as a guide to every physician in biomedical research involving human volunteers. They should be kept under review in the future.

It must be stressed that the standards as drafted are only a guide to physicians all over the world. Physicians are not relieved from criminal, civil and ethical responsibilities under the laws of their own countries.

I Basic Principles

Biomedical research involving human volunteers must conform to generally accepted scientific principles and should be based on adequately performed laboratory and animal experimentation and on a thorough knowledge of the scientific literature.

The design and performance of each experimental procedure involving human volunteers should be clearly formulated in an experimental protocol which should be transmitted for consideration, comment and guidance to a specially appointed committee independent of the investigator and the sponsor provided that this independent committee is in conformity with the laws and regulations of the country in which the research experiment is performed.

Biomedical research on human volunteers should be conducted only by scientifically qualified persons and under the supervision of a clinically competent medical person. The responsibility for the human volunteer must always rest with a medically qualified person and never rest on the volunteer of the research, even though the volunteer has given his or her consent.

Biomedical research involving human volunteers cannot legitimately be carried out unless the importance of the objective is in proportion to the inherent risk to the volunteer.

Every biomedical research project involving human volunteers should be preceded by careful assessment of predictable risks in comparison with foreseeable benefits to the volunteer or to others. Concern for the interest of the volunteer must always prevail over the interests of science and society.

The right of the research volunteer to safeguard his or her integrity must always be respected. Every precaution should be taken to respect the privacy of the volunteer and to minimise the impact of the study on the volunteer's physical and mental integrity and on the personality of the volunteer.

Physicians should abstain from engaging in research projects involving human volunteers unless they are satisfied that the hazards involved are believed to be predictable. Physicians should cease any investigation if the hazards are found to outweigh the potential benefits.

In publication of the results of his or her research, the physician is obliged to preserve the accuracy of the results. Reports on experimentation not in accordance with the principles laid down in this Declaration should not be accepted for publication.

In any research on human beings, each potential volunteer must be adequately informed of the aims, methods, anticipated benefits and potential hazards of the study and the discomfort it may entail. He or she should be informed that he or she is at liberty to abstain from participation in the study and that he or she is free to withdraw his or her consent to participation at any time. The physician should then obtain the volunteer's freely-given informed consent, preferably in writing.

When obtaining informed consent for the research project the physician should be particularly cautious if the volunteer is in a dependent relationship to him or her or may consent under duress. In that case the informed consent should be obtained by a physician who is not engaged in the investigation and who is completely independent of this official relationship.

In the case of legal incompetence, informed consent should be obtained from the legal guardian in accordance with national legislation. Where physical or mental incapacity makes it impossible to obtain informed consent, or when the volunteer is a minor, permission from the responsible relative replaces that of the volunteer in accordance with national legislation.

Whenever the minor child is in fact able to give consent, the minor's consent must be obtained in addition to the consent of the minor's legal guardian.

The research protocol should always contain a statement of the ethical considerations involved and should indicate that the principles enunciated in the present Declaration are complied with.

II Medical Research Combined With Professional Care (CLINICAL RESEARCH)

In the treatment of the sick person, the physician must be free to use a new diagnostic and therapeutic measure, if in his or her judgement it offers hope of saving life, re-establishing health or alleviating suffering.

The potential benefits, hazards and discomfort of a new method should be weighed against the advantages of the best current diagnostic and therapeutic methods.

In any medical study, every patient - including those of a control group, if any - should be assured of the best proven diagnostic and therapeutic method.

The refusal of the patient to participate in a study must never interfere with the physician-patient relationship.

If the physician considers it essential not to obtain informed consent, the specific reasons for this proposal should be stated in the experimental protocol for transmission to the independent committee.

The physician can combine medical research with professional care, the objective being the acquisition of new medical knowledge, only to the extent that medical research is justified by its potential diagnostic or therapeutic value for the patient

III Non-Therapeutic Biomedical Research Involving Human Volunteers (NON-CLINICAL BIOMEDICAL RESEARCH)

In the purely scientific application of medical research carried out on a human being, it is the duty of the physician to remain the protector of the life and health of that person on whom biomedical research is being carried out.

The volunteer should be volunteers - either healthy persons or patients for whom the experimental design is not related to the patient's illness.

The investigator or the investigating team should discontinue the research if in his/her or their judgement it may, if continued, be harmful to the individual.

In research on man, the interest of science and society should never take precedence over considerations related to the well-being of the volunteer.

The Validation of an Analytical Procedure for the Determination of Sulforaphane (SFN), Sulforaphane N-acetyl Cysteine (SFN-NAC) and Sulforaphane Glutathione (SFN-GSH) in Human Cerebrospinal Fluid (CSF) by LC-MS/MS, using Artificial CSF as a Surrogate Matrix

Study Sponsor	Evgen Ltd. 146 Brownlow Hill Liverpool L3 5RF
Bioanalytical Test Site	Alderley Analytical BioHub at Alderley Park Alderley Edge Cheshire SK10 4TG
Bioanalytical Project Leader	Alan Gibbs alan.gibbs@alderleyanalytical.com Telephone : +44 (0)1625 238610
Bioanalytical Method Title	Method for the Determination of SFN, SFN N-acetyl Cysteine and SFN Glutathione in CSF by LC-MS/MS
Alderley Analytical Study Number	0014/003
Alderley Analytical Method Number	0001/023
Species/Matrix/Stabiliser	Human / CSF / 0.5M Citric Acid
Stabiliser Concentration	300 µL of 0.5M citric acid per 17.7 mL of CSF
Surrogate Matrix	Artificial stabilised CSF
Validation Study Plan Client Issue Date	08 March 2016
Experimental Start Date	11 April 2016
Experimental Completion Date	10 May 2016
Report Issue Date	08 February 2017
Version	Final

MANAGEMENT STATEMENT

The bioanalytical validation report entitled “The Validation of an Analytical Procedure for the Determination of Sulforaphane (SFN), Sulforaphane N-acetyl Cysteine (SFN-NAC) and Sulforaphane Glutathione (SFN-GSH) in Human Cerebrospinal Fluid (CSF) by LC-MS/MS, using Artificial CSF as a Surrogate Matrix” has been reviewed and authorised by a member of the Alderley Analytical management team.

Elizabeth Thomas

Elizabeth Thomas
CEO

08 FEB 2017

Date

COMPLIANCE STATEMENT

Study Number: 0014/003

Study Title: The Validation of an Analytical Procedure for the Determination of Sulforaphane (SFN), Sulforaphane N-acetyl Cysteine (SFN-NAC) and Sulforaphane Glutathione (SFN-GSH) in Human Cerebrospinal Fluid (CSF) by LC-MS/MS, using Artificial CSF as a Surrogate Matrix

No formal claim of GLP compliance is required for work of this type, and no claim of compliance will be made for this validation.

However, all work carried out in this study was conducted in a Good Laboratory Practice (GLP) accredited laboratory in accordance with the OECD guidelines for GLP as incorporated into the United Kingdom statutory instrument for GLP 1999 No. 3106, as amended by statutory instrument 2004 No. 994.

All work was carried out in accordance with the Standard Operating Procedures of Alderley Analytical.

Alan Gibbs
Project Leader

08 Feb 2017
Date

QUALITY ASSURANCE STATEMENT

Study Number: 0014/003

Study Title: The Validation of an Analytical Procedure for the Determination of Sulforaphane (SFN), Sulforaphane N-acetyl Cysteine (SFN-NAC) and Sulforaphane Glutathione (SFN-GSH) in Human Cerebrospinal Fluid (CSF) by LC-MS/MS, using Artificial CSF as a Surrogate Matrix

Alderley Analytical QA has reviewed this report. The report is considered to accurately describe the methods and procedures used in the study and to accurately reflect the raw data of the study.

Inspections of this study were carried out on the following dates. Findings were reported to the Project Leader and to Management.

Object of Inspection	Inspection No.	Date of Inspection	Date reported to Project Leader and Management
Final Report Audit	QAAT014	06-19 Oct 2016	19 Oct 2016

Facilities relevant to this type of study are audited on an annual basis. Findings are reported to Management.

Jean Pearson
QA Representative

08 February 2017
Date

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1 SUMMARY

The objective of this study was to implement and validate a bioanalytical method for the quantitative analysis of SFN, SFN-NAC and SFN-GSH in Human Cerebrospinal Fluid.

Alderley Analytical has developed an LC-MS/MS assay for the measurement of SFN, SFN-NAC and SFN-GSH in human CSF (hCSF) samples, using artificial CSF (aCSF) as a surrogate matrix. This report details the accuracy and reproducibility of data obtained during the validation of the method.

The CSF standards and samples were extracted by a solid phase extraction method and analysed using a Waters I-Class UPLC, coupled to a Waters TQ-S Mass Spectrometer. UNIFI software (version number 1.7.1.0.0) was used to quantify peaks. Quantification was achieved using analyte peak area to internal standard (SFN-d_8 used as IS for SFN, SFN-NAC-d_8 used as IS for SFN-NAC and SFN-GSH) peak area ratios. Concentrations of the calibration curve standards, quality control samples and study samples were determined by the method of ($1/x^2$) weighted least squares linear regression.

The bioanalytical method (Alderley Analytical Method 0001/023) for the determination of SFN, SFN-NAC and SFN-GSH concentrations in CSF over ranges of 5 to 2000 ng/mL for SFN and SFN-NAC, and 10 to 2000 ng/mL for SFN-GSH, using a sample volume of 100 µL, can be found in Appendix 1.

The Certificates of Analysis for each compound used in the analysis are provided in Appendix 2.

The LC-MS/MS method for the determination of SFN, SFN-NAC and SFN-GSH concentrations in CSF was validated according to Alderley Analytical SOP L001 (Ref. 1), and Validation Plan Number 0014/003 (Appendix 3) for the given concentration ranges. The method validation results are summarised below.

Method Description	
Analyte	SFN, SFN-NAC, SFN-GSH
Matrix	Human Cerebrospinal Fluid, using Artificial Cerebrospinal Fluid as a surrogate matrix
Stabiliser	300 µL of 0.5M citric acid per 17.7 mL of CSF
Extraction Method	Solid Phase Extraction
Detection Method	LC-MS/MS
Sample Aliquot Volume	100 µL
Regression, Weighting	Linear, $1/x^2$
Quantification	Peak Area Ratios
Calibration Range	5 to 2000 ng/mL – SFN and SFN-NAC 10 to 2000 ng/mL – SFN-GSH

QC Levels	5, 15, 800, 1600 and 8000 ng/mL – SFN and SFN-NAC 10, 30, 800, 1600 and 8000 ng/mL – SFN-GSH		
Assay Performance			
Selectivity Blanks	SFN: ≤12.87% SFN IS: all 0.00% SFN-NAC: all 0.00% SFN-NAC IS: ≤3.58% SFN-GSH: ≤3.46% SFN-GSH IS: ≤3.49%		
Overall Precision and Accuracy		Precision (%CV)	Accuracy (%RE)
SFN	LLOQ:	10.24	94
	Low QC:	6.42	91
	Mid QC:	3.23	93
	High QC:	5.48	93
	ULOQ:	1.70	100
SFN-NAC	LLOQ:	13.53	103
	Low QC:	6.80	99
	Mid QC:	4.43	99
	High QC:	7.51	97
	ULOQ:	1.17	100
SFN-GSH	LLOQ:	10.03	107
	Low QC:	13.16	102
	Mid QC:	9.67	99
	High QC:	5.39	102
	ULOQ:	3.25	115
Dilution Integrity	SFN 10-fold: Precision 3.91%, Accuracy 95% SFN-NAC 10-fold: Precision 4.48%, Accuracy 93% SFN-GSH 10-fold: Precision 5.30%, Accuracy 91%		
Recovery	SFN:	95-102%	
	SFN-NAC:	56-77%	
	SFN-GSH:	27-41%	

	SFN IS:	91%
	SFN-NAC IS:	65%
	SFN-GSH IS:	65%
Matrix Effect Factor %CV	20.04% & 11.18% (SFN), 10.91% & 4.64% (SFN-NAC), 9.15% & 4.52% (SFN-GSH)	
Normalised Matrix Effect Factor %CV	5.50% & 6.67% (SFN), 7.54% & 4.24% (SFN-NAC), 5.75% & 2.69% (SFN-GSH)	
Effect of aCSF + 0.1% Blood	SFN:	Precision: 8.32% & 2.60%
		Accuracy: 106% & 102%
	SFN-NAC:	Precision: 4.73% & 1.60%
		Accuracy: 104% & 97%
	SFN-GSH:	Precision: 4.50% & 2.18%
		Accuracy: 88% & 106%
Effect of hCSF	SFN:	Precision: 4.86% & 2.68%
		Accuracy: 123% & 111%
	SFN-NAC:	Precision: 2.92% & 3.17%
		Accuracy: 90% & 91%
	SFN-GSH:	Precision: 5.16% & 4.84%
		Accuracy: 92% & 108%
Carryover	SFN:	0.00% to 17.47%
	SFN IS:	0.00%
	SFN-NAC:	0.00%
	SFN-NAC IS:	0.00%
	SFN-GSH:	0.00% to 7.17%
	SFN-GSH IS:	0.00%
Solution Stability (nominal 4°C)	SFN : 67 days	102%
	SFN-NAC : 69 days	96%
	SFN-GSH : 67 days	112%

Re-injection Reproducibility against original curve for partial batch re-injection	SFN Precision:	2.08% to 2.79%
	SFN Accuracy:	98% to 101%
	SFN-NAC Precision:	1.45% to 4.04%
	SFN-NAC Accuracy:	101% to 105%
	SFN-GSH Precision:	3.97% to 6.92%
	SFN-GSH Accuracy:	97% to 105%
Re-injection Reproducibility against re-injected curve for full batch re-injection	SFN Precision:	2.08% to 2.90%
	SFN Accuracy:	95% to 96%
	SFN-NAC Precision:	1.45% to 3.95%
	SFN-NAC Accuracy:	107% to 109%
	SFN-GSH Precision:	3.97% to 6.95%
	SFN-GSH Accuracy:	100% to 109%
Bench Top Stability on wet ice (aCSF)	SFN over 2.5 hours: Precision 1.39% to 6.76%, Accuracy 92% to 103%	
	SFN-NAC over 2.5 hours: Precision 1.36% to 5.15%, Accuracy 95% to 103%	
	SFN-GSH over 2.5 hours: Precision 0.94% to 6.70%, Accuracy 96% to 109%	
Bench Top Stability on wet ice (hCSF)	SFN over 2.5 hours: Precision 1.74% to 2.83%, Accuracy 112% to 116%	
	SFN-NAC over 2.5 hours: Precision 3.53% to 3.96%, Accuracy 84% to 100%	
	SFN-GSH over 2.5 hours: Precision 3.65% to 6.19%, Accuracy 84% to 94%	
Freeze/Thaw Stability at -80^{o}C/RT (aCSF)	SFN over 2 cycles: Precision 0.26% to 3.96%, Accuracy 91% to 98%	
	SFN-NAC over 2 cycles: Precision 1.21% to 3.02%, Accuracy 95% to 102%	
	SFN-GSH over 2 cycles: Precision 1.33% to 2.59%, Accuracy 97% to 119%	

Freeze/Thaw Stability at -80°C/RT (hCSF)	SFN over 2 cycles: Precision 3.34% to 4.07%, Accuracy 108% to 109%
	SFN-NAC over 2 cycles: Precision 1.11% to 8.59%, Accuracy 85% to 96%
	SFN-GSH over 2 cycles: Precision 1.13% to 4.90%, Accuracy 94% to 118%
Interference Screens	Maximum contribution: 12.69% (SFN not included in data as metabolites known to contain SFN)
Relative Retention Acceptance Range	acceptable

2 VALIDATION RESULTS

Selectivity

During Run 8, blank extracts generated from six individual lots of blank matrix were analysed. They were labelled as follows;

Matrix 1 = pooled human CSF (hCSF)
Matrix 2-4 = three lots of individual human CSF
Matrix 5 = individual human CSF + approximately 0.1% whole blood
Matrix 6 = stabilised artificial CSF (aCSF)

No significant interfering peaks (>20% of the lower limit of quantitation response) were observed at the retention time of SFN, SFN-NAC and SFN-GSH. In addition, no significant interfering peaks (>5% of mean internal standard response) were observed at the retention time of the internal standards. These results indicate that selectivity has been demonstrated for this assay in CSF (Tables 1a to 1f).

Linear Range and Response Function

A line was fitted through the data points of calibration standards prepared in aCSF by weighted linear regression (weight = $1/x^2$) for SFN, SFN-NAC and SFN-GSH of the concentration (x-axis) vs. peak area ratio (y-axis). SFN and SFN-NAC have a range of 5 to 2000 ng/mL, and SFN-GSH has a range of 10 to 2000 ng/mL (Figures 1a to 3c). Correlation coefficients for all calibration curves were at least 0.982 for SFN, SFN-NAC and SFN-GSH (Tables 2a to 2c).

Concentrations of the calibration standards were back-calculated and results are provided in Tables 3a to 3c. A calibration curve is acceptable provided that 75% of the back-calculated values for the standards analysed in the run do not deviate by more than ±15% at all concentrations, except at the LLOQ level where values can deviate up to ±20% of the nominal value. At least six concentration levels (including at least one replicate each of the LLOQ and ULOQ) must remain in the curve. All acceptance criteria were met.

Precision and Accuracy

Inter-run and intra-run precision and accuracy were determined by analysing four concentrations of QC samples prepared in aCSF in replicates of six over three separate batch runs on three different days.

- 5, 15, 800, and 1600 ng/mL for SFN and SFN-NAC
- 10, 30, 800, and 1600 ng/mL for SFN-GSH

For acceptance of the low, mid, and high QC levels, the precision around the mean value must not exceed 15% and the accuracy must be within ±15% of the nominal value. For acceptance at the LLOQ QC level, the precision around the mean value must not exceed 20% and the accuracy must be within ±20% of the nominal value.

Precision of the method, defined by the percent coefficient of variation (%CV) [standard deviation / mean × 100], was determined from the interpolated QC sample concentrations.

The intra-run precision for the QC samples at the LLOQ, low, mid, and high QC levels over the three precision and accuracy batch runs were all within batch acceptance criteria (Tables 4a to 4d).

The overall inter-run precision for the QC samples at the LLOQ, low, mid, and high QC levels over the three precision and accuracy batch runs was 10.24%, 6.42%, 3.23% and 5.48% respectively for SFN (Table 4e), 13.53%, 6.80%, 4.43% and 7.51% respectively for SFN-NAC (Table 4f), and 10.03%, 13.16%, 9.67% and 5.39% respectively for SFN-GSH (Table 4g including outliers, Table 4h with outliers removed).

The acceptance criteria for precision were met.

Accuracy of the method was defined by the percent relative error (%RE) [(mean observed concentration - nominal concentration) / nominal concentration × 100].

The intra-run accuracy for the QC samples at the LLOQ, low, mid, and high QC levels over the three precision and accuracy batch runs were all within batch acceptance criteria (Tables 4a to 4d) other than one level of one batch for SFN-GSH only (see section 3, point 1). Two results for SFN-GSH were removed from the calculations; these sample results were >1 SD from the appropriate intra-day mean value and >3 SD from the nominal concentrations, so are therefore considered to be statistical outliers.

The overall inter-run accuracy for the QC samples at the LLOQ, low, mid, and high QC levels over the three precision and accuracy batch runs was 94%, 91%, 93% and 93% respectively for SFN (Table 4e), 103%, 99%, 99% and 97% respectively for SFN-NAC (Table 4f), and 107%, 102%, 99% and 102% respectively for SFN-GSH (Table 4g including outliers, Table 4h with outliers removed).

The acceptance criteria for accuracy were met.

The upper limit of quantitation (ULOQ) of the assay was defined as the highest calibration standard concentration. Samples prepared at a concentration of 2000 ng/mL were analysed in replicates of six in Run 11 to yield precision and accuracy results of 1.70% and 100% respectively for SFN (Table 5a), 1.17% and 100% respectively for SFN-NAC (Table 5b) and 3.25% and 115% respectively for SFN-GSH (Table 5c).

The precision and accuracy are calculated from all replicates of the ULOQ sample. For acceptance, the precision around the mean value must not exceed 15% and the accuracy must be within ±15% of the nominal value.

The acceptance criteria for precision and accuracy were met.

Sensitivity

The lower limit of quantitation (LLOQ) of the assay was defined as the lowest calibration standard concentration. Samples prepared at a concentration of 5 ng/mL (for SFN and SFN-NAC) or 10 ng/mL (for SFN-GSH) were analysed in duplicate on each of three batch runs. The signal-to-noise ratio for each LLOQ standard was ≥5:1, and therefore met the acceptance criteria.

Evaluation of Large Run Size

Evaluation of Large Run Size was not performed during the validation (see section 3, point 2).

Dilution Integrity

QC samples prepared in aCSF containing 8000 ng/mL of SFN, SFN-NAC AND SFN-GSH were diluted 10-fold with blank matrix, extracted, and analysed in Run 9. A correction factor of 10 was applied to the concentration results of samples diluted 10-fold to yield precision and accuracy results of 3.91% and 95% respectively for SFN (Table 6a), 4.48% and 93% respectively for SFN-NAC (Table 6b) and 5.30% and 91% respectively for SFN-GSH (Table 6c).

The precision and accuracy are calculated from all replicates of the diluted QC sample. For acceptance, the precision around the mean value must not exceed 15% and the accuracy must be within ±15% of the nominal value.

The acceptance criteria for precision and accuracy were met. These results indicate that a sample with an analyte concentration higher than the upper limit of the calibration curve can be diluted 10-fold successfully.

Recovery (Extraction Efficiency)

Human Cerebrospinal Fluid samples at three concentrations in replicates of three were extracted and injected onto the LC-MS/MS system (*extracted* samples) in Run 13.

- 15, 800 and 1600 ng/mL for SFN and SFN-NAC
- 30, 800 and 1600 ng/mL for SFN-GSH

Solutions of SFN, SFN-NAC AND SFN-GSH spiked into extracted matrix blanks at concentrations representing 100% extraction recovery were also analysed by LC-MS/MS in replicates of three (*post-*

spiked samples). The comparison of the individual peak areas of extracted samples to the mean peak area of the post-spiked samples provided the extraction recovery results. The mean extraction recovery values ranged from 95% to 102% for SFN, from 56% to 77% for SFN-NAC and from 27% to 41% for SFN-GSH (Tables 7a to 7c).

The same experiment was used to determine the extraction recovery of the internal standard at the working concentration (50 ng/mL for internal standard in matrix) used for the assay. The overall mean extraction recovery was 91% for SFN IS, 65% for SFN-NAC IS and 65% for SFN-GSH IS (Tables 8a to 8c). Note that whilst SFN-NAC and SFN-GSH use the same internal standard, different values are used to calculate the results due to different Low QC2 samples being used.

Acceptance limits are not placed on recovery. The recovery of analytes and internal standards need not be 100%, but should be consistent across the concentration range. The recovery values obtained were sufficiently consistent across the concentration range to be considered acceptable (see section 3, point 3).

Matrix Effects

During Run 13, possible matrix effects were assessed in six individual lots of blank matrix. Each of the six lots of blank matrix (coding relating to blank matrices was as per the Selectivity section) were extracted in triplicate and then spiked with analyte and internal standard at concentrations equivalent to extracted low and high QC level samples (*spiked* samples with matrix present). Additionally, analytical solutions containing analyte and internal standard were prepared in triplicate in reconstitution solution, also at concentrations equivalent to extracted low and high QC samples (analytical solutions, no matrix present).

The matrix effect was calculated for each blank matrix lot using the Matrix Factor equation below:

Matrix Factor = (mean peak area in presence of matrix ions) / (mean peak area in absence of matrix ions)

A Matrix Factor (MF) of one indicates no matrix effect. An MF of less than one may indicate matrix suppression and an MF greater than one may indicate enhancement. An MF of one is not necessary, however the overall precision (%CV) of the Matrix Factors across all six lots should be ≤15%. If overall precision of the Matrix Factors is not within this limit, then the assessment is still acceptable, provided that the overall precision of the Normalized (internal standard adjusted) Matrix Factors across all six lots is ≤15%. The normalized matrix factor is calculated for each lot using the equation below:

Normalized Matrix Factor = (mean peak area ratio in presence of matrix ions) / (mean peak area ratio in absence of matrix ions)

The precision of the Matrix Factors for SFN at the low QC level (15 ng/mL) over the six lots of matrix was 20.04%, and the precision of the normalized matrix factors over the same six lots of matrix was 5.50% (Tables 9a and 10a), thus meeting the acceptance criteria of ≤15%. The precision of the Matrix Factors for SFN at the high QC level (1600 ng/mL) over the six lots of matrix was 11.18%, and the precision of the normalized matrix factors over the same six lots of matrix was 6.67% (Tables 9b and 10b), thus meeting the acceptance criteria of ≤15%.

The precision of the Matrix Factors for SFN-NAC at the low QC level (15 ng/mL) over the six lots of matrix was 10.91%, and the precision of the normalized matrix factors over the same six lots of matrix was 7.54% (Tables 9c and 10c), thus meeting the acceptance criteria of ≤15%. The precision of the Matrix Factors for SFN-NAC at the high QC level (1600 ng/mL) over the six lots of matrix was 4.64%, and the precision of the normalized matrix factors over the same six lots of matrix was 4.24% (Tables 9d and 10d), thus meeting the acceptance criteria of ≤15%.

The precision of the Matrix Factors for SFN-GSH at the low QC level (30 ng/mL) over the six lots of matrix was 9.15%, and the precision of the normalized matrix factors over the same six lots of matrix was 5.75% (Tables 9e and 10e), thus meeting the acceptance criteria of ≤15%. The precision of the Matrix Factors for SFN-GSH at the high QC level (1600 ng/mL) over the six lots of matrix was 4.52%, and the precision of the normalized matrix factors over the same six lots of matrix was 2.69% (Tables 9f and 10f), thus meeting the acceptance criteria of ≤15%.

Evaluation of Artificial CSF + 0.1% Blood

The effect resulting from the presence of blood in a CSF sample was assessed by preparing QC samples at low and high concentrations into artificial stabilised CSF containing 0.1% whole blood.

Six replicates of each aCSF + 0.1% blood sample, stored at the requisite temperature for at least 24 hours prior to use, were analysed along with a standard curve and run acceptance QCs in at least duplicate, prepared in artificial stabilised CSF.

For SFN, the precision and accuracy over the six lots of aCSF + 0.1% blood were 8.32% and 106% respectively at the low QC level, and 2.60% and 102% respectively at the high QC level (Table 11a), thus meeting the acceptance criteria of ≤15%.

For SFN-NAC, the precision and accuracy over the six lots of aCSF + 0.1% blood were 4.73% and 104% respectively at the low QC level, and 1.60% and 97% respectively at the high QC level (Table 11b), thus meeting the acceptance criteria of ≤15%.

For SFN-GSH, the precision and accuracy over the six lots of aCSF + 0.1% blood were 4.50% and 88% respectively at the low QC level, and 2.18% and 106% respectively at the high QC level (Table 11c), thus meeting the acceptance criteria of ≤15%.

Evaluation of Human CSF

The effect resulting from the use of human CSF was assessed by preparing QC samples at low and high concentrations into human CSF.

Six replicates of each hCSF sample, stored at the requisite temperature for at least 24 hours prior to use, were analysed along with a standard curve and run acceptance QCs in at least duplicate, prepared in artificial stabilised CSF.

For SFN, the precision and accuracy over the six lots of hCSF were 4.86% and 123% respectively at the low QC level, and 2.68% and 111% respectively at the high QC level (Table 12a), thus the acceptance criteria of ≤15% was not met for accuracy at one level only (see section 3, point 4).

For SFN-NAC, the precision and accuracy over the six lots of hCSF were 2.92% and 90% respectively at the low QC level, and 3.17% and 91% respectively at the high QC level (Table 12b), thus meeting the acceptance criteria of ≤15%.

For SFN-GSH, the precision and accuracy over the six lots of hCSF were 5.16% and 92% respectively at the low QC level, and 4.84% and 108% respectively at the high QC level (Table 12c), thus meeting the acceptance criteria of ≤15%.

Carryover Evaluation

Blank samples were analysed after each ULOQ standard (2000 ng/mL for SFN, SFN-NAC and SFN-GSH) to assess carryover. The response (peak area) for the blank samples must be ≤20% of the mean LLOQ response to be acceptable. No significant carryover was observed for SFN, SFN-NAC and SFN-GSH (Tables 13a to 13c).

In addition, the carryover response for the internal standard must be ≤5% of the mean internal standard response to be acceptable. No significant carryover was observed for the internal standard (Tables 14a to 14c).

Stability

Stability in matrix is proven (for all assessments, except solution stability) provided that at least 2/3 of the stability quality control samples at each level (as indicated in the stability sections below) are within ±15% of nominal concentrations and the mean concentrations do not deviate from the nominal concentrations by more than ±15% relative error.

Stability in analytical solutions is proven provided that the percent difference of the stored solution compared to the fresh solution is within ±10%.

Stability was determined for the following conditions:

2.1.1 Solution Stability (nominal 4ºC/-20ºC and Room Temperature)

2.1.1.1 Nominal 4ºC/-20ºC

Stock stability testing at a nominal 4ºC/-20ºC was performed as part of method validation study number 0014/001 (Ref. 3). The relevant results are included in Appendix 4.

2.1.2 Extract Stability

Extract Stability is determined over the anticipated time that an entire run may be stored prior to analysis. It is established by storing an entire run (at a minimum, blanks, calibration standards, and triplicate low, medium and high QC samples) for the desired time at the requisite temperature prior to injection. Extract stability is calculated from the completion of sample preparation until injection of the first QC sample or calibration standard.

This testing was not performed as part of this validation (see section 3, point 5).

2.1.3 Re-Injection Reproducibility / Autosampler Stability

Re-injection reproducibility / autosampler stability is determined over the anticipated time that a batch run may be stored prior to its re-injection. It is established by analysing a previously injected batch run (at a minimum, blanks, standards, and triplicate low, medium and high QC samples) for the desired time after storage at the requisite autosampler tray temperature. Re-injection reproducibility storage time is calculated from the original injection of the first QC sample or calibration standard until re-injection of the first QC sample or calibration standard.

The re-injected QC samples were compared to both the original calibration curve and to the re-injected calibration curve (Tables 15a to 15c). This is to confirm partial batch re-injection and full batch re-injection, respectively. Acceptance criteria is as per standard batch acceptance.

All acceptance criteria were met, therefore both partial batches and full batches of extracted samples of SFN, SFN-NAC and SFN-GSH can be re-injected, following storage on the autosampler of up to 24 hours.

2.1.4 Benchtop Stability

QC samples at three concentrations in both artificial and human cerebrospinal fluid were stored for 2.5 hours on wet ice prior to aliquoting and extracting in replicates of three.

- 15, 1600 and 8000 ng/mL for SFN and SFN-NAC
- 30, 1600 and 8000 ng/mL for SFN-GSH

The results indicate that SFN, SFN-NAC and SFN-GSH are stable in both artificial and human cerebrospinal fluid for at least 2.5 hours on wet ice (see section 3, point 6). The accuracy after 2.5

hours on wet ice in aCSF ranged from 92% to 103% for SFN, 95% to 103% for SFN-NAC and 96% to 109% for SFN-GSH (Tables 16a to 16c). The accuracy after 2.5 hours on wet ice in hCSF ranged from 112% to 116% for SFN, 84% to 100% for SFN-NAC and 84% to 94% for SFN-GSH (Tables 17a to 17c).

2.1.5 Freeze/Thaw Stability

QC samples at three concentrations in both artificial and human cerebrospinal fluid were prepared and subjected to two freeze (-80°C)/thaw cycles (see section 3, point 7). At the end of the second freeze/thaw cycle, the samples were aliquoted in triplicate, extracted, and analysed.

- 15, 1600 and 8000 ng/mL for SFN and SFN-NAC
- 30, 1600 and 8000 ng/mL for SFN-GSH

The results indicate that SFN, SFN-NAC and SFN-GSH are stable in both artificial and human cerebrospinal fluid for at least two freeze (-80°C)/thaw cycles prior to analysis (see section 3, point 8). The accuracy after two freeze (-80°C)/thaw cycles in aCSF ranged from 91% to 98% for SFN, 95% to 102% for SFN-NAC and 97% to 119% for SFN-GSH (Tables 18a to 18c). The accuracy after two freeze (-80°C)/thaw cycles in hCSF ranged from 108% to 109% for SFN, 85% to 96% for SFN-NAC and 94% to 118% for SFN-GSH (Tables 19a to 19c).

2.1.6 Interference Screens

SFN, SFN-NAC and SFN-GSH were analysed independently to monitor the contribution of each analyte on the other. This was performed at the Low QC and High QC level for each analyte.

Independent samples containing each separate analyte were prepared at the Low QC and High QC levels (without addition of internal standard) in artificial CSF and analysed in triplicate. These were analysed for the other analytes (i.e. samples spiked with SFN will be analysed for SFN-NAC and SFN-GSH, and so on).

The maximum amount of interference noted in any one sample was 12.69%, other than for SFN (Tables 20a to 20c). It was previously known that the pure standards of the metabolites contained SFN, therefore the standard interference screen acceptance criteria of <20% of the mean Low QC level did not apply to that analyte. All interference screen results were therefore acceptable.

2.1.7 Long Term Storage Stability

A separate study, to investigate frozen stability over three time points, has been agreed. This will therefore be covered in a separate report (study number 0014/004).

2.1.8 Retention Time

Absolute retention was monitored during each validation run by comparing the retention times of the analyte and internal standard between the beginning and end of each run. These varied by no more than 10% for any run.

2.1.9 Typical Chromatograms

Typical chromatograms of a blank sample, and at the lower and upper limits of quantification, are shown in Figures 4 to 7.

2.1.10 Analytical Notes

A summary of all runs performed in the validation of the bioanalytical method for SFN, SFN-NAC and SFN-GSH in CSF is provided below:

Run No. (Date)	Description / Comments	Results
Run 1 11 Apr 2016	• n/a	Not used as a result of updated method
Run 2 14 Apr 2016	• n/a	Not used as a result of updated method
Run 3 15 Apr 2016	• n/a	Not used as a result of updated method
Run 4 18 Apr 2016	• n/a	Not used as a result of updated method
Run 5 19 Apr 2016	• n/a	Not used as a result of updated method
Run 6 25 Apr 2016	• Precision & Accuracy Run 1 • Carryover	Pass
Run 7 26 Apr 2016	• Precision & Accuracy Run 2 • Carryover	Pass
Run 8 27 Apr 2016	• Precision & Accuracy Run 3 • Carryover • Selectivity	Pass
Run 9 28 Apr 2016	• Dilution QCs	Pass
Run 10 29 Apr 2016	• Reinjection Reproducibility (24 hours)	Pass

Run 11 03 May 2016	• Precision & Accuracy at ULOQ • aCSF + 0.1% Blood Testing • hCSF Testing	Pass
Run 12 03 May 2016	• n/a	Fail – Additional on-system testing, insufficient stability
Run 13 04 May 2016	• Recovery • Matrix Effects	Pass
Run 14 06 May 2016	• Interference Screens	Pass
Run 15 09 May 2016	• Bench-Top Stability	Pass
Run 16 10 May 2016	• Freeze-Thaw Cycling	Pass

3 DEVIATIONS

1) One intra-day result for SFN-GSH on one batch run at one concentration only gave a 116% accuracy, outside standard acceptance of 85-115%. This is considered to be acceptable.

2) It is expected that this method will only be used to analyse small numbers of clinical samples, which will be received and analysed on an ongoing basis. Therefore, evaluation of a large batch size was not considered to be required as part of this validation.

3) Post-spiked samples' precision values for SFN-NAC and SFN-GSH recovery testing were approximately 1-2% greater than standard acceptance criteria. This is not considered to be significant.

4) Human CSF accuracy results were outside standard acceptance criteria (123%), at the low QC level only, for SFN only. As the high QC level results for SFN were acceptable, and results in human CSF for the other two analytes were also acceptable, it is thought that this relates to a preparative error rather than a significant bioanalytical issue. This is therefore considered to be acceptable.

5) It is expected that all batch runs during sample analysis will be analysed immediately upon their preparation, therefore separate pre-analysis storage testing was not performed as part of this validation.

6) Bench-top stability testing was performed on wet ice over a period of 2.5 hours, rather than the 4 hours stated in the study plan. It will be ensured that this will be the maximum time for which samples will be stored on bench-top prior to extraction. For bench-top stability in hCSF, all three analytes had one accuracy result of ±16% at one concentration only, outside

standard acceptance of 85-115%. As all analytes' overall mean accuracy was within this range, this is considered to be acceptable.

7) Freeze-thaw cycling was performed over two, rather than three cycles. It will be ensured all clinical samples will undergo no more than two freeze-thaw cycles prior to analysis.

8) Freeze-thaw cycling results for SFN-GSH at the high QC level only were outside standard acceptance criteria for both aCSF and hCSF (119% and 118%). As this was seen in both matrices at one level only, and overall mean accuracy was within the standard acceptance range on both occasions, it is thought that this relates to a preparative error rather than a significant bioanalytical issue. This is therefore considered to be acceptable.

4 CONCLUSIONS

The LC-MS/MS analytical method for the determination of SFN, SFN-NAC and SFN-GSH concentrations in human CSF, using artificial CSF as a surrogate matrix, over ranges of 5 to 2000 ng/mL for SFN and SFN-NAC, and 10 to 2000 ng/mL for SFN-GSH, using a sample volume of 100 µL, has been demonstrated to be precise and accurate, and is suitable for the analysis of clinical study samples.

5 ARCHIVE PROCEDURE

All records of the study including the validation plan, raw data and approved final report are archived at Alderley Analytical, approved and documented according to Alderley Analytical SOP QA009 (Ref. 2). Records will be retained for a period of two years from report finalisation, after which time the Sponsor will be contacted to determine requirements for further storage, return or destruction of materials. No materials will be destroyed without written instruction from the Sponsor.

6 REFERENCES

1. Alderley Analytical SOP L001: Validation of Bioanalytical Methods.
2. Alderley Analytical SOP QA009: Archiving Procedures / Records Management.
3. Alderley Analytical Study No. 0014/001: The Validation of an Analytical Procedure for the Determination of Sulforaphane (SFN), Sulforaphane N-acetyl Cysteine (SFN-NAC) and Sulforaphane Glutathione (SFN-GSH) in Human K_2 EDTA Stabilised Plasma by LC-MS/MS.

7 TABLES

Table 1a Selectivity for SFN

Sample ID	SFN Peak Area	% Response compared to Mean
LLOQ QC1	18923	
LLOQ QC2	18990	
LLOQ QC3	21434	
LLOQ QC4	20038	
LLOQ QC5	19223	
LLOQ QC6	21869	
Mean	20080	
Matrix 1	0	0.00%
Matrix 2	0	0.00%
Matrix 3	2012	10.02%
Matrix 4	2585	12.87%
Matrix 5	0	0.00%
Matrix 6	2180	10.86%

Table 1b Selectivity for SFN Internal Standard

Sample ID	SFN IS Peak Area	% Response compared to IS Mean
LLOQ QC1	522167	
LLOQ QC2	548787	
LLOQ QC3	549905	
LLOQ QC4	559015	
LLOQ QC5	534934	
LLOQ QC6	585325	
Mean	550022	
Matrix 1	0	0.00%
Matrix 2	0	0.00%
Matrix 3	0	0.00%
Matrix 4	0	0.00%
Matrix 5	0	0.00%
Matrix 6	0	0.00%

Table 1c Selectivity for SFN-NAC

Sample ID	SFN-NAC Peak Area	% Response compared to Mean
LLOQ QC1	33717	
LLOQ QC2	39590	
LLOQ QC3	39895	
LLOQ QC4	48812	
LLOQ QC5	42928	
LLOQ QC6	39196	
Mean	40690	
Matrix 1	0	0.00%
Matrix 2	0	0.00%
Matrix 3	0	0.00%
Matrix 4	0	0.00%
Matrix 5	0	0.00%
Matrix 6	0	0.00%

Table 1d Selectivity for SFN-NAC Internal Standard

Sample ID	SFN-NAC IS Peak Area	% Response compared to IS Mean
LLOQ QC1	297131	
LLOQ QC2	299396	
LLOQ QC3	317563	
LLOQ QC4	306223	
LLOQ QC5	278658	
LLOQ QC6	280457	
Mean	296571	
Matrix 1	10629	3.58%
Matrix 2	0	0.00%
Matrix 3	0	0.00%
Matrix 4	0	0.00%
Matrix 5	0	0.00%
Matrix 6	0	0.00%

Table 1e Selectivity for SFN-GSH

Sample ID	SFN-GSH Peak Area	% Response compared to Mean
LLOQ QC1	251939	
LLOQ QC2	257623	
LLOQ QC3	251691	
LLOQ QC4	268555	
LLOQ QC5	246069	
LLOQ QC6	228207	
Mean	250681	
Matrix 1	0	0.00%
Matrix 2	0	0.00%
Matrix 3	3820	1.52%
Matrix 4	0	0.00%
Matrix 5	8681	3.46%
Matrix 6	0	0.00%

Table 1f Selectivity for SFN-GSH Internal Standard

Sample ID	SFN-GSH IS Peak Area	% Response compared to IS Mean
LLOQ QC1	309300	
LLOQ QC2	303793	
LLOQ QC3	316975	
LLOQ QC4	293182	
LLOQ QC5	303600	
LLOQ QC6	302447	
Mean	304883	
Matrix 1	10629	3.49%
Matrix 2	0	0.00%
Matrix 3	0	0.00%
Matrix 4	0	0.00%
Matrix 5	0	0.00%
Matrix 6	0	0.00%

Table 2a Standard Curve Parameters for SFN

Run Number	Gradient	Intercept	Correlation (R^2)
Run 6	5.37×10^{-3}	6.86×10^{-3}	0.995270
Run 7	5.86×10^{-3}	5.65×10^{-3}	0.992853
Run 8	6.13×10^{-3}	6.10×10^{-3}	0.989477
Run 9	8.20×10^{-3}	3.29×10^{-3}	0.998728
Run 10	7.91×10^{-3}	7.54×10^{-3}	0.990996
Run 11	5.46×10^{-3}	7.33×10^{-3}	0.996198
Run 13	N/A	N/A	N/A
Run 14	N/A	N/A	N/A
Run 15	5.51×10^{-3}	5.90×10^{-3}	0.994734
Run 16	5.81×10^{-3}	5.76×10^{-3}	0.994188

Runs 1-5 and 12 standard curve summary data not tabulated as the runs were not used as part of the validation, as described in Section 2.1.10.

No calibration curve was run with Runs 13 and 14 as results used peak areas only.

Table 2b Standard Curve Parameters for SFN-NAC

Run Number	Gradient	Intercept	Correlation (R^2)
Run 6	2.42×10^{-2}	-1.03×10^{-2}	0.993750
Run 7	2.64×10^{-2}	-9.52×10^{-3}	0.990379
Run 8	2.38×10^{-2}	9.04×10^{-3}	0.993938
Run 9	2.57×10^{-2}	1.18×10^{-2}	0.992599
Run 10	2.47×10^{-2}	2.79×10^{-3}	0.989107
Run 11	2.62×10^{-2}	1.68×10^{-2}	0.997285
Run 13	N/A	N/A	N/A
Run 14	N/A	N/A	N/A
Run 15	2.60×10^{-2}	3.11×10^{-3}	0.994905
Run 16	2.68×10^{-2}	9.06×10^{-3}	0.996980

Runs 1-5 and 12 standard curve summary data not tabulated as the runs were not used as part of the validation, as described in Section 2.1.10.

No calibration curve was run with Runs 13 and 14 as results used peak areas only.

Table 2c Standard Curve Parameters for SFN-GSH

Run Number	Gradient	Intercept	Correlation (R^2)
Run 6	5.97×10^{-2}	7.03×10^{-2}	0.988169
Run 7	8.53×10^{-2}	-2.77×10^{-2}	0.989047
Run 8	8.98×10^{-2}	-5.32×10^{-2}	0.996910
Run 9	9.97×10^{-2}	2.22×10^{-1}	0.988412
Run 10	9.65×10^{-2}	2.32×10^{-1}	0.982919
Run 11	6.40×10^{-2}	5.07×10^{-3}	0.993318
Run 13	N/A	N/A	N/A
Run 14	N/A	N/A	N/A
Run 15	6.05×10^{-2}	-9.34×10^{-2}	0.988898
Run 16	5.86×10^{-2}	-6.77×10^{-2}	0.993799

Runs 1-5 and 12 standard curve summary data not tabulated as the runs were not used as part of the validation, as described in Section 2.1.10.

No calibration curve was run with Runs 13 and 14 as results used peak areas only.

Table 3a Standard Curve Back Calculated Concentrations of SFN

Run Number[#]	Nominal Concentration (ng/mL)								
	5.00	10.00	25.00	50.00	100.00	250.00	500.00	1000.00	2000.00
Run 6	4.68	10.14	26.89	49.63	106.60	256.46	517.42	970.99	1906.04
	5.38	9.26							1858.39
Run 7	5.81	9.76	25.37	49.05	103.49	251.46	509.76	1026.72	1951.15
	4.26	9.88							1925.08
Run 8	5.79	10.62	26.39	52.92	103.95	250.49	509.24	962.52	1921.49
	4.14	9.29							1852.85
Run 9	4.97	9.38	25.82	50.85	103.04	251.97	499.97	981.90	2031.91
	5.14	9.97							1912.45
Run 10	5.58	10.67	26.81	53.82	104.25	257.41	474.03	926.03	1996.14
	4.40	8.93							1901.61
Run 11	5.34	9.69	27.49	50.41	103.96	254.57	495.27	991.89	1928.73
	4.84	9.13							1938.85
Run 15	4.78	9.54	27.83	49.60	103.66	256.26	493.60	998.01	1891.19
	5.46	9.03							1995.97
Run 16	5.14	11.31	25.57	54.94	95.46	248.91	490.91	955.47	1995.98
	4.68	9.18							1959.64
Number	16	16	8	8	8	8	8	8	16
Mean	5.02	9.74	26.52	51.40	103.05	253.44	498.78	976.69	1935.47
SD	0.52	0.67	0.90	2.20	3.25	3.15	13.59	30.39	50.84
CV	10.34%	6.91%	3.38%	4.28%	3.15%	1.24%	2.72%	3.11%	2.63%
Accuracy	100.49%	97.36%	106.09%	102.81%	103.05%	101.38%	99.76%	97.67%	96.77%

Key

[#] Runs 1-5 and 12 standard curve concentration data not tabulated as the runs were not used as part of the validation, as described in Section 2.1.10.

Table 3b Standard Curve Back Calculated Concentrations of SFN-NAC

Run Number[#]	Nominal Concentration (ng/mL)								
	5.00	10.00	25.00	50.00	100.00	250.00	500.00	1000.00	2000.00
Run 6	4.83	9.67	27.31	51.21	101.06	252.13	509.63	947.76	1762.95
	4.82	11.31							1976.71
Run 7	5.61	8.55	26.93	54.44	98.48	248.81	500.92	994.26	1939.80
	4.44	10.78							1889.82
Run 8	5.13	10.54	24.73	50.80	102.01	255.12	507.11	996.87	1819.23
	4.42	11.26							1888.06
Run 9	4.34	9.84	25.01	54.34	104.21	251.57	491.29	1005.61	1862.17
	5.74	9.63							1964.07
Run 10	4.08	9.52	24.81	47.77	96.66	257.61	516.55	997.67	1980.43
	5.98	10.38							2064.84
Run 11	4.83	9.66	24.44	49.45	100.91	261.64	517.16	1004.91	1898.72
	5.42	9.41							2063.75
Run 15	4.53	10.10	27.92	51.09	98.54	257.59	497.97	988.79	1900.65
	5.43	9.55							1905.57
Run 16	4.85	9.91	25.37	54.71	98.58	259.46	500.17	991.74	1880.62
	4.99	10.47							1855.84
Number	16	16	8	8	8	8	8	8	16
Mean	4.97	10.04	25.82	51.73	100.06	255.49	505.10	990.95	1915.83
SD	0.55	0.72	1.35	2.55	2.43	4.37	9.14	18.41	80.57
CV	11.04%	7.20%	5.25%	4.93%	2.43%	1.71%	1.81%	1.86%	4.21%
Accuracy	99.30%	100.36%	103.26%	103.45%	100.06%	102.20%	101.02%	99.10%	95.79%

Key

[#] Runs 1-5 and 12 standard curve concentration data not tabulated as the runs were not used as part of the validation, as described in Section 2.1.10.

Table 3c Standard Curve Back Calculated Concentrations of SFN-GSH

Run Number[#]	Nominal Concentration (ng/mL)							
	10.00	25.00	50.00	100.00	250.00	500.00	1000.00	2000.00
Run 6	8.35	28.20	49.97	99.33	233.22	519.43	948.80	2013.79
	11.17							*
Run 7	10.99	26.05	54.80	95.86	220.75	491.11	979.13	2262.41
	8.74							1909.79
Run 8	10.58	23.61	52.38	92.60	257.66	499.87	1050.04	2022.55
	9.60							1943.74
Run 9	8.24	*	55.75	98.65	248.98	473.62	960.88	2036.15
	11.56							1993.10
Run 10	8.18	28.75	46.10	94.89	243.74	458.12	956.10	2082.37
	11.45							2255.44
Run 11	8.89	23.98	51.05	100.62	239.46	492.37	986.23	2143.25
	11.24							*
Run 15	10.64	25.77	43.55	89.99	233.20	474.93	1044.44	2282.34
	9.62							2209.17
Run 16	9.89	25.15	48.39	90.42	233.40	498.09	1048.23	2257.69
	10.27							*
Number	16	7	8	8	8	8	8	13
Mean	9.96	25.93	50.25	95.30	238.80	488.44	996.73	2108.60
SD	1.20	1.96	4.17	4.06	11.35	19.00	43.79	132.70
CV	12.01%	7.54%	8.30%	4.26%	4.75%	3.89%	4.39%	6.29%
Accuracy	99.63%	103.72%	100.50%	95.30%	95.52%	97.69%	99.67%	105.43%

Key

[#] Runs 1-5 and 12 standard curve concentration data not tabulated as the runs were not used as part of the validation, as described in Section 2.1.10.

* Calibration standard removed from the regression equation as the % deviation exceeded the acceptance criteria of ±15% (±20% at LLOQ).

Table 4a Intra-Run Quality Control Sample Concentrations of SFN

Nominal Conc.	LLOQ QC1 5.00 ng/mL	Low QC2 15.00 ng/mL	Mid QC3 800.00 ng/mL	High QC4 1600.00 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.	Calc. Conc.
Run 6	4.20	14.56	706.52	1485.87
(P&A Run 1)	4.36	13.91	714.04	1364.63
	3.71	12.88	741.39	1357.30
	4.47	11.79	732.46	1372.96
	4.49	14.85	722.70	1352.21
	4.85	12.67	713.23	1391.77
Mean	4.34	13.44	721.72	1387.46
SD	0.38	1.19	13.15	50.17
CV	8.72%	8.87%	1.82%	3.62%
Accuracy	87%	90%	90%	87%
Run 7	4.22	14.22	751.37	1539.59
(P&A Run 2)	4.53	14.68	773.27	1545.49
	4.54	12.42	779.14	1553.97
	5.89	13.54	791.47	1550.06
	4.93	14.68	755.68	1550.57
	4.65	13.52	764.91	1559.21
Mean	4.79	13.84	769.30	1549.82
SD	0.58	0.87	15.03	6.77
CV	12.20%	6.26%	1.95%	0.44%
Accuracy	96%	92%	96%	97%
Run 8	4.92	13.86	741.96	1524.49
(P&A Run 3)	4.65	12.80	764.00	1477.80
	5.36	13.83	730.54	1595.40
	4.85	13.21	731.47	1487.62
	4.87	14.38	759.16	1543.04
	5.10	14.09	756.88	1506.93
Mean	4.96	13.70	747.34	1522.55
SD	0.25	0.58	14.64	42.89
CV	4.94%	4.26%	1.96%	2.82%
Accuracy	99%	91%	93%	95%

Table 4b Intra-Run Quality Control Sample Concentrations of SFN-NAC

Nominal Conc.	LLOQ QC1 5.00 ng/mL	Low QC2 15.00 ng/mL	Mid QC3 800.00 ng/mL	High QC4 1600.00 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.	Calc. Conc.
Run 6	3.96	15.23	698.44	1507.93
(P&A Run 1)	4.54	16.18	757.54	1370.30
	5.88	14.46	761.67	1309.78
	5.49	15.49	785.69	1405.26
	3.87	12.30	758.32	1461.84
	5.28	15.93	770.24	1435.02
Mean	4.84	14.93	755.32	1415.02
SD	0.84	1.42	29.78	69.85
CV	17.31%	9.51%	3.94%	4.94%
Accuracy	97%	100%	94%	88%
Run 7	5.87	14.29	784.04	1558.09
(P&A Run 2)	5.68	15.72	782.31	1552.10
	5.18	15.58	785.94	1592.57
	5.10	14.29	763.68	1525.98
	4.52	14.03	793.31	1619.60
	5.29	14.95	803.39	1670.94
Mean	5.27	14.81	785.45	1586.55
SD	0.47	0.72	13.19	52.76
CV	9.00%	4.87%	1.68%	3.33%
Accuracy	105%	99%	98%	99%
Run 8	4.39	14.21	808.88	1673.45
(P&A Run 3)	5.17	14.13	827.50	1535.07
	4.90	14.52	835.60	1637.67
	6.31	13.85	830.34	1716.63
	6.09	14.53	810.52	1692.19
	5.49	16.40	837.26	1605.95
Mean	5.39	14.61	825.02	1643.49
SD	0.73	0.91	12.38	66.06
CV	13.51%	6.26%	1.50%	4.02%
Accuracy	108%	97%	103%	103%

Table 4c Intra-Run Quality Control Sample Concentrations of SFN-GSH

Nominal Conc.	LLOQ QC1 10.00 ng/mL	Low QC2 30.00 ng/mL	Mid QC3 800.00 ng/mL	High QC4 1600.00 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.	Calc. Conc.
Run 6	11.63	36.09	794.65	1679.98
(P&A Run 1)	11.36	30.89	840.21	1478.83
	10.82	35.81	908.69	1462.41
	14.62	33.77	956.66	1707.15
	11.98	37.98	909.02	1744.08
	12.32	44.36	860.06	1685.40
Mean	12.12	36.48	878.21	1626.31
SD	1.33	4.55	57.95	122.79
CV	10.95%	12.48%	6.60%	7.55%
Accuracy	121%	122%	110%	102%
Run 7	9.79	30.91	761.02	1614.15
(P&A Run 2)	10.62	29.90	772.40	1638.27
	10.10	32.80	695.37	1678.89
	11.29	34.64	773.24	1675.70
	12.59	30.25	719.57	1774.82
	10.11	28.16	707.38	1602.97
Mean	10.75	31.11	738.16	1664.13
SD	1.04	2.29	34.78	62.47
CV	9.70%	7.37%	4.71%	3.75%
Accuracy	107%	104%	92%	104%
Run 8	9.66	26.22	803.06	1709.66
(P&A Run 3)	10.03	27.55	763.92	1588.78
	9.43	25.54	720.25	1652.02
	10.79	25.33	714.68	1695.98
	9.61	28.12	760.16	1590.27
	8.99	25.97	754.88	1499.51
Mean	9.75	26.46	752.82	1622.71
SD	0.61	1.13	32.31	78.96
CV	6.26%	4.26%	4.29%	4.87%
Accuracy	98%	88%	94%	101%

Table 4d **Intra-Run Quality Control Sample Concentrations of SFN-GSH (outliers removed)**

Nominal Conc.	LLOQ QC1 10.00 ng/mL	Low QC2 30.00 ng/mL	Mid QC3 800.00 ng/mL	High QC4 1600.00 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.	Calc. Conc.
Run 6	11.63	36.09	794.65	1679.98
(P&A Run 1)	11.36	30.89	840.21	1478.83
	10.82	35.81	908.69	1462.41
	14.62*	33.77	956.66	1707.15
	11.98	37.98	909.02	1744.08
	12.32	44.36*	860.06	1685.40
Mean	11.62	34.91	878.21	1626.31
SD	0.58	2.70	57.95	122.79
CV	4.96%	7.73%	6.60%	7.55%
Accuracy	116%	116%	110%	102%
Run 7	9.79	30.91	761.02	1614.15
(P&A Run 2)	10.62	29.90	772.40	1638.27
	10.10	32.80	695.37	1678.89
	11.29	34.64	773.24	1675.70
	12.59	30.25	719.57	1774.82
	10.11	28.16	707.38	1602.97
Mean	10.75	31.11	738.16	1664.13
SD	1.04	2.29	34.78	62.47
CV	9.70%	7.37%	4.71%	3.75%
Accuracy	107%	104%	92%	104%
Run 8	9.66	26.22	803.06	1709.66
(P&A Run 3)	10.03	27.55	763.92	1588.78
	9.43	25.54	720.25	1652.02
	10.79	25.33	714.68	1695.98
	9.61	28.12	760.16	1590.27
	8.99	25.97	754.88	1499.51
Mean	9.75	26.46	752.82	1622.71
SD	0.61	1.13	32.31	78.96
CV	6.26%	4.26%	4.29%	4.87%
Accuracy	98%	88%	94%	101%

* QC sample result removed from calculations due to being a statistical outlier

Table 4e Inter-Run Quality Control Sample Concentrations of SFN

Nominal Conc. (ng/mL)	Number	Mean	SD	CV	Accuracy
5.00	18	4.70	0.48	10.24%	94%
15.00	18	13.66	0.88	6.42%	91%
800.00	18	746.12	24.10	3.23%	93%
1600.00	18	1486.61	81.43	5.48%	93%

Table 4f Inter-Run Quality Control Sample Concentrations of SFN-NAC

Nominal Conc. (ng/mL)	Number	Mean	SD	CV	Accuracy
5.00	18	5.17	0.70	13.53%	103%
15.00	18	14.78	1.01	6.80%	99%
800.00	18	788.59	34.92	4.43%	99%
1600.00	18	1548.35	116.28	7.51%	97%

Table 4g Inter-Run Quality Control Sample Concentrations of SFN-GSH

Nominal Conc. (ng/mL)	Number	Mean	SD	CV	Accuracy
10.00	18	10.87	1.40	12.84%	109%
30.00	18	31.35	5.08	16.20%	104%
800.00	18	789.73	76.37	9.67%	99%
1600.00	18	1637.72	88.25	5.39%	102%

Table 4h Inter-Run Quality Control Sample Concentrations of SFN-GSH (outliers removed)

Nominal Conc. (ng/mL)	Number	Mean	SD	CV	Accuracy
10.00	17	10.65	1.07	10.03%	107%
30.00	17	30.58	4.02	13.16%	102%
800.00	18	789.73	76.37	9.67%	99%
1600.00	18	1637.72	88.25	5.39%	102%

Table 5a Upper Limit of Quantitation for SFN

Nominal Conc.	ULOQ QC5 2000.00 ng/mL
	Calc. Conc.
Run 11	2052.06
	1992.45
	2002.80
	1969.68
	1985.77
	1953.84
Mean	1992.77
SD	33.80
CV	1.70%
Accuracy	100%

Table 5b Upper Limit of Quantitation for SFN-NAC

Nominal Conc.	ULOQ QC5 2000.00 ng/mL
	Calc. Conc.
Run 11	1962.62
	2016.43
	2002.83
	2017.88
	1969.84
	1997.51
Mean	1994.52
SD	23.37
CV	1.17%
Accuracy	100%

Table 5c Upper Limit of Quantitation for SFN-GSH

Nominal Conc.	ULOQ QC5 2000.00 ng/mL
	Calc. Conc.
Run 11	2336.84
	2392.26
	2340.16
	2230.05
	2219.89
	2226.69
Mean	2290.98
SD	74.40
CV	3.25%
Accuracy	115%

Table 6a Dilution Integrity (10-fold) for SFN

Nominal Conc.	Dil QC 8000.00 ng/mL
	Calc. Conc.
Run 9	7182.31
	7965.28
	7734.52
	7414.46
	7499.47
	7871.16
Mean	7611.20
SD	297.76
CV	3.91%
Accuracy	95%

Table 6b Dilution Integrity (10-fold) for SFN-NAC

Nominal Conc.	Dil QC 8000.00 ng/mL
	Calc. Conc.
Run 9	7161.85
	7975.31
	7166.51
	7714.79
	7244.04
	7553.99
Mean	7469.42
SD	334.79
CV	4.48%
Accuracy	93%

Table 6c Dilution Integrity (10-fold) for SFN-GSH

Nominal Conc.	Dil QC 8000.00 ng/mL
	Calc. Conc.
Run 9	7103.00
	7928.71
	6931.98
	7495.51
	7251.80
	6930.76
Mean	7273.62
SD	385.29
CV	5.30%
Accuracy	91%

Table 7a Recovery (Extraction Efficiency) for SFN

Nominal Conc.	Low QC2 15.00 ng/mL		Mid QC3 800.00 ng/mL		High QC4 1600.00 ng/mL	
	Peak Areas		Peak Areas		Peak Areas	
	Post Spiked	Extracted	Post Spiked	Extracted	Post Spiked	Extracted
Run 13	38072	38087	1897239	1955262	3929017	3770366
	40842	41599	1968212	1921232	3542619	3542645
	39783	41219	2086821	2001292	3820315	3423307
Mean	39565	40302	1984090	1959262	3763984	3578773
SD	1398	1928	95783	40179	199263	176328
CV	3.53%	4.78%	4.83%	2.05%	5.29%	4.93%
Recovery		102%		99%		95%

Table 7b Recovery (Extraction Efficiency) for SFN-NAC

Nominal Conc.	Low QC2 15.00 ng/mL		Mid QC3 800.00 ng/mL		High QC4 1600.00 ng/mL	
	Peak Areas		Peak Areas		Peak Areas	
	Post Spiked	Extracted	Post Spiked	Extracted	Post Spiked	Extracted
Run 13	232363	128457	10414756	7992349	18687094	14071366
	249479	139641	10836101	8007427	17335861	13871977
	246413	142198	11096040	8466046	18055172	13633432
Mean	242752	136765	10782299	8155274	18026043	13858925
SD	9127	7308	343814	269242	676087	219259
CV	3.76%	5.34%	3.19%	3.30%	3.75%	1.58%
Recovery		56%		76%		77%

Table 7c Recovery (Extraction Efficiency) for SFN-GSH

Nominal Conc.	Low QC2 30.00 ng/mL		Mid QC3 800.00 ng/mL		High QC4 1600.00 ng/mL	
	Peak Areas		Peak Areas		Peak Areas	
	Post Spiked	Extracted	Post Spiked	Extracted	Post Spiked	Extracted
Run 13	253555	78336	46644050	18818982	88897351	36991263
	263955	65052	49634606	18313830	83152238	35649496
	248022	66511	50722141	19172679	88811907	33233128
Mean	255177	69966	49000266	18768497	86953832	35291296
SD	8090	7285	2111752	431644	3292554	1904501
CV	3.17%	10.41%	4.31%	2.30%	3.79%	5.40%
Recovery		27%		38%		41%

Table 8a Recovery (Extraction Efficiency) for SFN Internal Standard

	IS In-Sample Conc. 50 ng/mL	
	IS Peak Areas	
	Post Spiked	Extracted
Low QC2	477902	429370
	477665	428232
	462726	419113
Mid QC3	444837	396500
	410632	403286
	439987	444194
High QC4	460106	404009
	434556	382383
	442236	392964
Mean	450072	411117
SD	21747	20202
CV	4.83%	4.91%
Recovery		91%

Table 8b Recovery (Extraction Efficiency) for SFN-NAC Internal Standard

	IS In-Sample Conc. 50 ng/mL	
	IS Peak Areas	
	Post Spiked	Extracted
Low QC2	702845	379509
	704348	380996
	740837	396357
Mid QC3	559700	427709
	572847	404418
	588205	424312
High QC4	540620	365126
	493878	373470
	489794	366381
Mean	599230	390920
SD	94027	23667
CV	15.69%	6.05%
Recovery		65%

Table 8c Recovery (Extraction Efficiency) for SFN-GSH Internal Standard

	IS In-Sample Conc. 50 ng/mL	
	IS Peak Areas	
	Post Spiked	Extracted
Low QC2	760774	376019
	736142	391571
	708964	399040
Mid QC3	559700	427709
	572847	404418
	588205	424312
High QC4	540620	365126
	493878	373470
	489794	366381
Mean	605658	392005
SD	103321	23718
CV	17.06%	6.05%
Recovery		65%

Table 9a Matrix Effects at 15 ng/mL of SFN (Low QC)

	Peak Area						
	Solution	Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6
Low QC1	65214	21820	49369	54349	38970	39444	47034
Low QC2	48462	22591	33588	33796	35333	32533	40210
Low QC3	43507	22118	24459	33787	30102	28618	37611
Mean	52394	22176	35805	40644	34802	33532	41619
CV	21.71%	1.75%	35.20%	29.20%	12.81%	16.35%	11.69%
Matrix Factor		0.4233	0.6834	0.7757	0.6642	0.6400	0.7943
Overall Precision							20.04%

Table 9b Matrix Effects at 1600 ng/mL of SFN (High QC)

	Peak Area						
	Solution	Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6
High QC1	3808418	1927255	3084863	2723257	2473716	2517807	2820952
High QC2	3187527	2163583	2674439	2565547	2193984	2539397	2964613
High QC3	3243873	2182702	2575724	2649405	2386150	2653433	2785350
Mean	3413273	2091180	2778342	2646070	2351284	2570212	2856972
CV	10.06%	6.80%	9.72%	2.98%	6.09%	2.84%	3.32%
Matrix Factor		0.6127	0.8140	0.7752	0.6889	0.7530	0.8370
Overall Precision							11.18%

Table 9c Matrix Effects at 15 ng/mL of SFN-NAC (Low QC)

	Peak Area						
	Solution	Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6
Low QC1	134504	157314	158668	185823	154239	175804	183973
Low QC2	171849	170037	151022	183962	189421	160487	207195
Low QC3	153579	151146	125404	196368	190652	167664	195626
Mean	153311	159499	145032	188718	178104	167985	195598
CV	12.18%	6.04%	12.01%	3.55%	11.61%	4.56%	5.94%
Matrix Factor		1.0404	0.9460	1.2309	1.1617	1.0957	1.2758
Overall Precision							10.91%

Table 9d Matrix Effects at 1600 ng/mL of SFN-NAC (High QC)

	Peak Area						
	Solution	Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6
High QC1	13904961	12758528	12801548	13094616	12853907	12627203	13369210
High QC2	14260472	14304996	12078609	12999648	12230080	13428834	14569333
High QC3	14323102	15510058	12499325	14739536	14263841	14849424	13966086
Mean	14162845	14191194	12459827	13611267	13115943	13635154	13968210
CV	1.59%	9.72%	2.91%	7.19%	7.94%	8.25%	4.30%
Matrix Factor		1.0020	0.8798	0.9611	0.9261	0.9627	0.9863
Overall Precision							4.64%

Table 9e Matrix Effects at 30 ng/mL of SFN-GSH (Low QC)

	Peak Area						
	Solution	Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6
Low QC1	108066	178253	179025	160314	161815	178361	214757
Low QC2	112859	204779	161857	157498	189814	181544	196168
Low QC3	116554	212665	200371	182731	189298	196481	237723
Mean	112493	198566	180418	166848	180309	185462	216216
CV	3.78%	9.08%	10.69%	8.29%	8.88%	5.22%	9.63%
Matrix Factor		1.7651	1.6038	1.4832	1.6028	1.6487	1.9220
Overall Precision							9.15%

Table 9f Matrix Effects at 1600 ng/mL of SFN-GSH (High QC)

	Peak Area						
	Solution	Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6
High QC1	53267140	70252499	74513374	70005281	68081585	67823241	67061570
High QC2	53842979	79814648	71719863	68877233	63614190	72021762	70887144
High QC3	54510516	78244062	67784233	70038635	71402704	76365019	64906176
Mean	53873545	76103736	71339157	69640383	67699493	72070007	67618296
CV	1.16%	6.74%	4.74%	0.95%	5.77%	5.93%	4.48%
Matrix Factor		1.4126	1.3242	1.2927	1.2566	1.3378	1.2551
Overall Precision							4.52%

Table 10a Normalised Matrix Effects at 15 ng/mL of SFN (Low QC)

	Peak Area Ratio						
	Solution	Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6
Low QC1	0.118	0.105	0.115	0.105	0.108	0.099	0.100
Low QC2	0.095	0.105	0.119	0.097	0.105	0.106	0.090
Low QC3	0.101	0.118	0.098	0.096	0.105	0.102	0.097
Mean	0.104	0.109	0.110	0.100	0.106	0.102	0.096
CV	11.39%	6.64%	10.28%	4.86%	1.77%	3.46%	5.36%
Normalised Matrix Factor		1.0451	1.0574	0.9544	1.0128	0.9765	0.9160
Overall Precision							5.50%

Table 10b Normalised Matrix Effects at 1600 ng/mL of SFN (High QC)

	Peak Area Ratio						
	Solution	Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6
High QC1	9.424	9.315	10.675	11.147	9.640	10.084	8.964
High QC2	9.182	10.248	11.254	10.085	9.054	10.694	9.148
High QC3	9.270	9.652	9.926	10.094	11.127	10.092	8.232
Mean	9.292	9.738	10.618	10.442	9.940	10.290	8.782
CV	1.32%	4.86%	6.27%	5.85%	10.75%	3.40%	5.52%
Normalised Matrix Factor		1.0480	1.1427	1.1237	1.0698	1.1074	0.9451
Overall Precision							6.67%

Table 10c Normalised Matrix Effects at 15 ng/mL of SFN-NAC (Low QC)

	Peak Area Ratio						
	Solution	Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6
Low QC1	0.325	0.288	0.262	0.267	0.266	0.286	0.342
Low QC2	0.355	0.290	0.347	0.325	0.318	0.276	0.360
Low QC3	0.365	0.297	0.305	0.329	0.336	0.313	0.362
Mean	0.349	0.292	0.304	0.307	0.307	0.292	0.355
CV	6.03%	1.46%	14.02%	11.34%	11.86%	6.41%	3.18%
Normalised Matrix Factor		0.8371	0.8735	0.8813	0.8794	0.8370	1.0180
Overall Precision							7.54%

Table 10d Normalised Matrix Effects at 1600 ng/mL of SFN-NAC (High QC)

	Peak Area Ratio						
	Solution	Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6
High QC1	38.257	30.849	30.509	33.624	31.742	29.026	34.320
High QC2	35.780	31.295	30.692	30.504	29.086	32.344	37.200
High QC3	36.208	30.501	31.371	33.515	34.093	34.469	32.002
Mean	36.748	30.882	30.857	32.548	31.640	31.946	34.508
CV	3.60%	1.29%	1.47%	5.44%	7.92%	8.59%	7.55%
Normalised Matrix Factor		0.8404	0.8397	0.8857	0.8610	0.8693	0.9390
Overall Precision							4.24%

Table 10e Normalised Matrix Effects at 30 ng/mL of SFN-GSH (Low QC)

	Peak Area Ratio						
	Solution	Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6
Low QC1	0.246	0.352	0.425	0.328	0.319	0.344	0.379
Low QC2	0.250	0.306	0.314	0.343	0.332	0.307	0.336
Low QC3	0.286	0.338	0.388	0.384	0.329	0.332	0.358
Mean	0.261	0.332	0.376	0.352	0.327	0.328	0.358
CV	8.46%	7.12%	14.98%	8.25%	2.07%	5.67%	5.90%
Normalised Matrix Factor		1.2725	1.4410	1.3501	1.2523	1.2564	1.3712
Overall Precision							5.75%

Table 10f Normalised Matrix Effects at 1600 ng/mL of SFN-GSH (High QC)

	Peak Area Ratio						
	Solution	Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6
High QC1	146.557	169.864	177.582	179.756	168.125	155.906	172.156
High QC2	135.093	174.612	182.241	161.622	151.289	173.466	180.999
High QC3	137.798	153.868	170.124	159.255	170.663	177.263	148.725
Mean	139.816	166.115	176.649	166.878	163.359	168.878	167.293
CV	4.29%	6.54%	3.46%	6.72%	6.45%	6.75%	9.97%
Normalised Matrix Factor		1.1881	1.2634	1.1936	1.1684	1.2079	1.1965
Overall Precision							2.69%

Table 11a SFN in aCSF + 0.1% Whole Blood

Nominal Conc.	Low QC2 15.00 ng/mL	High QC4 1600.00 ng/mL
	Calc. Conc.	Calc. Conc.
	16.64	1667.39
	15.07	1647.91
	17.62	1582.12
	13.86	1632.68
	16.39	1661.39
	15.61	1566.82
Mean	15.87	1626.39
SD	1.32	42.23
CV	8.32%	2.60%
Accuracy	106%	102%

Table 11b SFN-NAC in aCSF + 0.1% Whole Blood

Nominal Conc.	Low QC2 15.00 ng/mL	High QC4 1600.00 ng/mL
	Calc. Conc.	Calc. Conc.
	16.32	1568.93
	15.45	1557.32
	14.99	1588.61
	14.92	1570.45
	15.30	1542.95
	16.73	1517.06
Mean	15.62	1557.55
SD	0.74	24.95
CV	4.73%	1.60%
Accuracy	104%	97%

Table 11c SFN-GSH in aCSF + 0.1% Whole Blood

Nominal Conc.	Low QC2 30.00 ng/mL	High QC4 1600.00 ng/mL
	Calc. Conc.	Calc. Conc.
	27.91	1687.10
	27.46	1759.93
	24.65	1717.63
	26.38	1676.83
	26.41	1708.27
	25.63	1653.20
Mean	26.40	1700.49
SD	1.19	37.05
CV	4.50%	2.18%
Accuracy	88%	106%

Table 12a SFN in hCSF

Nominal Conc.	Low QC2 15.00 ng/mL	High QC4 1600.00 ng/mL
	Calc. Conc.	Calc. Conc.
	18.15	1779.06
	18.75	1781.21
	18.01	1704.71
	19.62	1774.27
	18.92	1851.68
	17.02	1754.47
Mean	18.41	1774.23
SD	0.89	47.53
CV	4.86%	2.68%
Accuracy	123%	111%

Table 12b SFN-NAC in hCSF

Nominal Conc.	Low QC2 15.00 ng/mL	High QC4 1600.00 ng/mL
	Calc. Conc.	Calc. Conc.
	13.62	1429.45
	13.76	1472.69
	13.31	1461.28
	13.02	1533.69
	13.28	1425.41
	14.12	1404.32
Mean	13.52	1454.47
SD	0.39	46.13
CV	2.92%	3.17%
Accuracy	90%	91%

Table 12c SFN-GSH in hCSF

Nominal Conc.	Low QC2 15.00 ng/mL	High QC4 1600.00 ng/mL
	Calc. Conc.	Calc. Conc.
	28.66	1673.27
	26.10	1723.51
	28.78	1780.28
	29.05	1829.37
	25.94	1732.19
	26.89	1590.39
Mean	27.57	1721.50
SD	1.42	83.34
CV	5.16%	4.84%
Accuracy	92%	108%

Table 13a Carryover Assessment for SFN

Sample ID	Peak Area	% Response of Mean LLOQ	Peak Area	% Response of Mean LLOQ	Peak Area	% Response of Mean LLOQ
	Run 6		Run 7		Run 8	
STD1A	20843	n/a	20670	n/a	24594	n/a
STD1B	17738	n/a	11967	n/a	18886	n/a
Mean LLOQ Response	19290		16318		21740	
Blank 1	3126	16.21%	2757	16.89%	2112	9.72%
Blank 2	0	0.00%	854	5.23%	3798	17.47%

Table 13b Carryover Assessment for SFN-NAC

Sample ID	Peak Area	% Response of Mean LLOQ	Peak Area	% Response of Mean LLOQ	Peak Area	% Response of Mean LLOQ
	Run 6		Run 7		Run 8	
STD1A	27609	n/a	28833	n/a	44185	n/a
STD1B	26172	n/a	31445	n/a	30494	n/a
Mean LLOQ Response	26890		30139		37340	
Blank 1	0	0.00%	0	0.00%	0	0.00%
Blank 2	0	0.00%	0	0.00%	0	0.00%

Table 13c Carryover Assessment for SFN-GSH

Sample ID	Peak Area	% Response of Mean LLOQ	Peak Area	% Response of Mean LLOQ	Peak Area	% Response of Mean LLOQ
	Run 6		Run 7		Run 8	
STD2A	202441	n/a	214517	n/a	326423	n/a
STD2B	223637	n/a	203482	n/a	231155	n/a
Mean LLOQ Response	213039		209000		278789	
Blank 1	13478	6.33%	13224	6.33%	16832	6.04%
Blank 2	14753	6.92%	0	0.00%	19981	7.17%

Table 14a Carryover Assessment for SFN IS

Sample ID	IS Peak Area	% Response of Mean IS in LLOQ	IS Peak Area	% Response of Mean IS in LLOQ	IS Peak Area	% Response of Mean IS in LLOQ
	Run 6		Run 7		Run 8	
STD1A	651420	n/a	520075	n/a	591608	n/a
STD1B	496029	n/a	390609	n/a	599493	n/a
Mean IS in LLOQ Response	573725		455342		595551	
Blank 1	0	0.00%	0	0.00%	0	0.00%
Blank 2	0	0.00%	0	0.00%	0	0.00%

Table 14b Carryover Assessment for SFN-NAC IS

Sample ID	IS Peak Area	% Response of Mean IS in LLOQ	IS Peak Area	% Response of Mean IS in LLOQ	IS Peak Area	% Response of Mean IS in LLOQ
	Run 6		Run 7		Run 8	
STD1A	259045	n/a	207963	n/a	337026	n/a
STD1B	246121	n/a	292279	n/a	266869	n/a
Mean IS in LLOQ Response	252583		250121		301948	
Blank 1	0	0.00%	0	0.00%	0	0.00%
Blank 2	0	0.00%	0	0.00%	0	0.00%

Table 14c Carryover Assessment for SFN-GSH IS

Sample ID	IS Peak Area	% Response of Mean IS in LLOQ	IS Peak Area	% Response of Mean IS in LLOQ	IS Peak Area	% Response of Mean IS in LLOQ
	Run 6		Run 7		Run 8	
STD2A	356124	n/a	235953	n/a	363698	n/a
STD2B	303476	n/a	283640	n/a	285511	n/a
Mean IS in LLOQ Response	329800		259797		324605	
Blank 1	0	0.00%	0	0.00%	0	0.00%
Blank 2	0	0.00%	0	0.00%	0	0.00%

Table 15a 24 Hour Re-Injection Reproducibility and Autosampler Stability for SFN

	Re-Run QCs (Run 10) Calculated Against	
	Original Cal Curve Run 9	Re-Injected Cal Curve Run 10
Low QC 15 ng/mL	14.97 15.06 14.94 15.24 16.06 15.04	13.97 14.06 13.93 14.23 15.02 14.03
Mean SD CV Accuracy	15.22 0.42 2.79% 101%	14.21 0.41 2.90% 95%
Mid QC 800 ng/mL	785.14 771.53 800.23 797.14 785.35 819.59	760.98 747.77 775.61 772.61 761.18 794.39
Mean SD CV Accuracy	793.16 16.48 2.08% 99%	768.76 15.98 2.08% 96%
High QC 1600 ng/mL	1525.37 1530.42 1550.80 1590.78 1615.92 1602.07	1478.94 1483.84 1503.61 1542.39 1566.77 1553.34
Mean SD CV Accuracy	1569.23 38.71 2.47% 98%	1521.48 37.55 2.47% 95%

Table 15b 24 Hour Re-Injection Reproducibility and Autosampler Stability for SFN-NAC

	Re-Run QCs (Run 10) Calculated Against	
	Original Cal Curve Run 9	Re-Injected Cal Curve Run 10
Low QC 15 ng/mL	14.60 14.31 15.68 15.40 15.26 15.87	15.55 15.24 16.67 16.39 16.24 16.87
Mean SD CV Accuracy	15.19 0.61 4.04% 101%	16.16 0.64 3.95% 108%
Mid QC 800 ng/mL	840.50 804.67 830.26 830.26 878.04 838.95	874.64 837.37 863.98 863.99 913.68 873.02
Mean SD CV Accuracy	837.11 23.82 2.85% 105%	871.11 24.77 2.84% 109%
High QC 1600 ng/mL	1651.60 1660.30 1611.82 1684.72 1660.78 1646.21	1718.32 1727.38 1676.94 1752.78 1727.87 1712.72
Mean SD CV Accuracy	1652.57 23.93 1.45% 103%	1719.34 24.89 1.45% 107%

Table 15c 24 Hour Re-Injection Reproducibility and Autosampler Stability for SFN-GSH

	Re-Run QCs (Run 10) Calculated Against	
	Original Cal Curve Run 9	Re-Injected Cal Curve Run 10
Low QC 30 ng/mL	31.34 27.96 25.96 28.38 29.73 30.87	32.29 28.79 26.73 29.24 30.63 31.81
Mean SD CV Accuracy	29.04 2.01 6.92% 97%	29.91 2.08 6.95% 100%
Mid QC 800 ng/mL	781.47 729.84 795.68 806.76 843.77 873.00	807.69 754.32 822.38 833.83 872.09 902.30
Mean SD CV Accuracy	805.09 49.84 6.19% 101%	832.10 51.52 6.19% 104%
High QC 1600 ng/mL	1640.91 1636.30 1606.79 1739.72 1775.89 1711.34	1696.08 1691.32 1660.81 1798.22 1835.61 1768.88
Mean SD CV Accuracy	1685.16 66.91 3.97% 105%	1741.82 69.16 3.97% 109%

Table 16a 2.5 Hour Benchtop Stability for SFN in aCSF

	Low QC 15 ng/mL	High QC 1600 ng/mL	Dil QC 8000 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.
	14.20	1539.58	8320.62
	13.99	1551.86	8098.60
	13.10	1733.70	8257.20
Mean	13.76	1608.38	8225.47
SD	0.58	108.71	114.36
CV	4.25%	6.76%	1.39%
Accuracy	92%	101%	103%

Table 16b 2.5 Hour Benchtop Stability for SFN-NAC in aCSF

	Low QC 15 ng/mL	High QC 1600 ng/mL	Dil QC 8000 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.
	15.14	1521.89	8000.07
	14.23	1534.86	8430.00
	15.77	1494.40	8224.40
Mean	15.04	1517.05	8218.16
SD	0.78	20.66	215.03
CV	5.15%	1.36%	2.62%
Accuracy	100%	95%	103%

Table 16c 2.5 Hour Benchtop Stability for SFN-GSH in aCSF

	Low QC 30 ng/mL	High QC 1600 ng/mL	Dil QC 8000 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.
	30.84	1726.74	7886.39
	27.20	1755.14	7811.55
	27.96	1726.90	7456.60
Mean	28.67	1736.26	7718.18
SD	1.92	16.35	229.60
CV	6.70%	0.94%	2.97%
Accuracy	96%	109%	96%

Table 17a 2.5 Hour Benchtop Stability for SFN in hCSF

	Low QC 15 ng/mL	High QC 1600 ng/mL	Dil QC 8000 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.
	16.69	1775.18	9064.00
	17.33	1834.50	9326.66
	17.65	1787.95	9463.89
Mean	17.22	1799.21	9284.85
SD	0.49	31.22	203.20
CV	2.83%	1.74%	2.19%
Accuracy	115%	112%	116%

Table 17b 2.5 Hour Benchtop Stability for SFN-NAC in hCSF

	Low QC 15 ng/mL	High QC 1600 ng/mL	Dil QC 8000 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.
	14.92	1388.03	8264.88
	13.79	1326.02	8042.20
	14.47	1295.35	7648.96
Mean	14.39	1336.46	7985.34
SD	0.57	47.21	311.87
CV	3.96%	3.53%	3.91%
Accuracy	96%	84%	100%

Table 17c 2.5 Hour Benchtop Stability for SFN-GSH in hCSF

	Low QC 30 ng/mL	High QC 1600 ng/mL	Dil QC 8000 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.
	27.32	1570.97	7159.70
	25.46	1500.16	6385.47
	25.99	1434.51	6517.74
Mean	26.26	1501.88	6687.64
SD	0.96	68.25	414.13
CV	3.65%	4.54%	6.19%
Accuracy	88%	94%	84%

Table 18a 2-Cycle Freeze (-80°C) /Thaw Stability for SFN in aCSF

	Low QC 15 ng/mL	High QC 1600 ng/mL	Dil QC 8000 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.
	13.92	1522.03	7862.26
	13.00	1506.33	7894.11
	13.96	1477.24	7855.56
Mean	13.62	1501.87	7870.64
SD	0.54	22.73	20.60
CV	3.96%	1.51%	0.26%
Accuracy	91%	94%	98%

Table 18b 2-Cycle Freeze (-80°C) /Thaw Stability for SFN-NAC in aCSF

	Low QC 15 ng/mL	High QC 1600 ng/mL	Dil QC 8000 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.
	15.30	1474.13	8115.01
	14.63	1565.50	8258.23
	14.84	1512.80	8069.42
Mean	14.93	1517.48	8147.56
SD	0.34	45.86	98.52
CV	2.30%	3.02%	1.21%
Accuracy	100%	95%	102%

Table 18c 2-Cycle Freeze (-80°C) /Thaw Stability for SFN-GSH in aCSF

	Low QC 30 ng/mL	High QC 1600 ng/mL	Dil QC 8000 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.
	29.15	1850.77	8675.05
	28.33	1927.60	8904.02
	29.65	1942.89	8832.09
Mean	29.04	1907.09	8803.72
SD	0.67	49.37	117.09
CV	2.30%	2.59%	1.33%
Accuracy	97%	119%	110%

Table 19a 2-Cycle Freeze (-80°C) /Thaw Stability for SFN in hCSF

	Low QC 15 ng/mL	High QC 1600 ng/mL	Dil QC 8000 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.
	16.14	1818.38	8849.51
	15.59	1698.53	8400.92
	16.91	1722.48	8949.32
Mean	16.21	1746.46	8733.25
SD	0.66	63.42	292.10
CV	4.07%	3.63%	3.34%
Accuracy	108%	109%	109%

Table 19b 2-Cycle Freeze (-80°C) /Thaw Stability for SFN-NAC in hCSF

	Low QC 15 ng/mL	High QC 1600 ng/mL	Dil QC 8000 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.
	15.77	1347.00	8003.71
	13.51	1376.53	7465.90
	13.80	1367.36	7433.52
Mean	14.36	1363.63	7634.38
SD	1.23	15.11	320.26
CV	8.59%	1.11%	4.19%
Accuracy	96%	85%	95%

Table 19c 2-Cycle Freeze (-80°C) /Thaw Stability for SFN-GSH in hCSF

	Low QC 30 ng/mL	High QC 1600 ng/mL	Dil QC 8000 ng/mL
	Calc. Conc.	Calc. Conc.	Calc. Conc.
	33.07	1883.67	7805.27
	30.80	1913.95	7199.21
	30.14	1872.70	7478.03
Mean	31.33	1890.11	7494.17
SD	1.54	21.36	303.35
CV	4.90%	1.13%	4.05%
Accuracy	104%	118%	94%

Table 20a Interference Screens Relative to SFN

Sample ID	SFN Peak Area	% Response compared to Mean
Low QC Q1A	100129	
Low QC Q1B	101336	
Low QC Q1C	106556	
Mean	102674	
SFN-NAC Low QC		
Q1A	624	0.61%
Q1B	597	0.58%
Q1C	0	0.00%
SFN-GSH Low QC		
Q1A	4568	4.45%
Q1B	0	0.00%
Q1C	5902	5.75%
SFN-NAC High QC		
Q1A	194151	189.09%
Q1B	210463	204.98%
Q1C	219728	214.01%
SFN-GSH High QC		
Q1A	396121	385.81%
Q1B	412685	401.94%
Q1C	405888	395.32%

Table 20b Interference Screens Relative to SFN-NAC

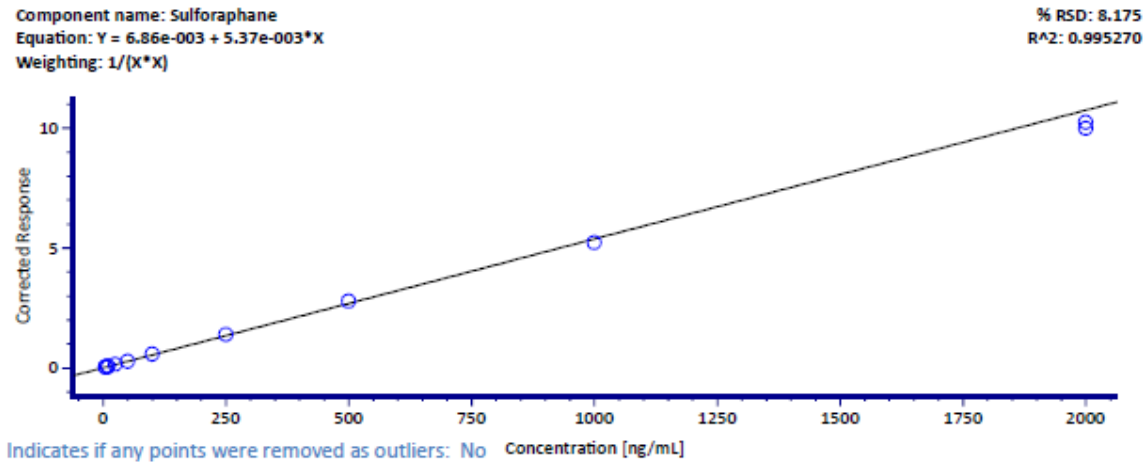
Sample ID	SFN-NAC Peak Area	% Response compared to Mean
Low QC Q1A	61815	
Low QC Q1B	61538	
Low QC Q1C	62656	
Mean	62003	
SFN Low QC		
Q1A	0	0.00%
Q1B	0	0.00%
Q1C	0	0.00%
SFN-GSH Low QC		
Q1A	0	0.00%
Q1B	0	0.00%
Q1C	0	0.00%
SFN High QC		
Q1A	0	0.00%
Q1B	0	0.00%
Q1C	5044	8.14%
SFN-GSH High QC		
Q1A	5448	8.79%
Q1B	0	0.00%
Q1C	0	0.00%

Table 20c Interference Screens Relative to SFN-GSH

Sample ID	SFN-GSH Peak Area	% Response compared to Mean
Low QC Q1A	69478	
Low QC Q1B	83480	
Low QC Q1C	76819	
Mean	76592	
SFN Low QC		
Q1A	0	0.00%
Q1B	5819	7.60%
Q1C	0	0.00%
SFN-NAC Low QC		
Q1A	0	0.00%
Q1B	5067	6.62%
Q1C	0	0.00%
SFN High QC		
Q1A	6952	9.08%
Q1B	8354	10.91%
Q1C	0	0.00%
SFN-NAC High QC		
Q1A	9719	12.69%
Q1B	7210	9.41%
Q1C	0	0.00%

8 FIGURES

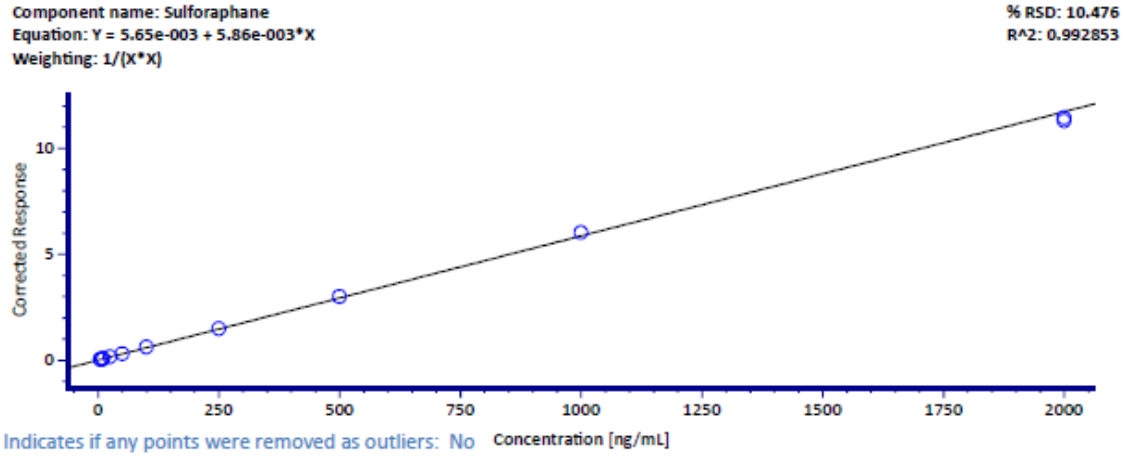
Figure 1a Standard Curve of SFN in Artificial Cerebrospinal Fluid, P&A Run 1



Component name: Sulforaphane

.	Sample name	Description	Rep	Sample Position	Response	IS Response	Response Ratio	Known Conc	Calc Conc	% Dev	Manual
1	6STD1A	STD	1	2:A,12	20843	651420	0.032	5.00	4.68	-6.41	None
2	6STD1B	STD	1	2:B,1	17738	496029	0.036	5.00	5.38	7.60	None
3	6STD2A	STD	1	2:B,2	38958	635346	0.061	10.00	10.14	1.38	None
4	6STD2B	STD	1	2:B,3	29583	522616	0.057	10.00	9.26	-7.39	None
5	6STD3	STD	1	2:B,4	82163	542957	0.151	25.00	26.89	7.57	None
6	6STD4	STD	1	2:B,5	152506	557739	0.273	50.00	49.63	-0.75	None
7	6STD5	STD	1	2:B,6	348947	602137	0.580	100.00	106.60	6.60	None
8	6STD6	STD	1	2:B,7	818377	591103	1.384	250.00	256.46	2.58	None
9	6STD7	STD	1	2:B,8	1432582	514145	2.786	500.00	517.42	3.48	None
10	6STD8	STD	1	2:B,9	2933264	561629	5.223	1000.00	970.99	-2.90	None
11	6STD9A	STD	1	2:B,10	5235129	510959	10.246	2000.00	1906.04	-4.70	None
12	6STD9B	STD	1	2:B,11	4343153	434762	9.990	2000.00	1858.39	-7.08	None

Figure 1b Standard Curve of SFN in Artificial Cerebrospinal Fluid, P&A Run 2

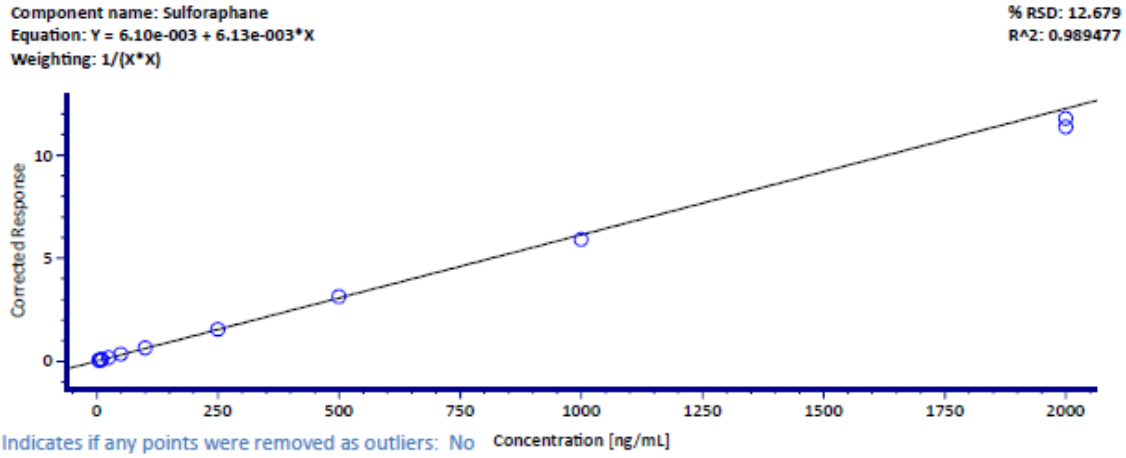


Indicates if any points were removed as outliers: No

Component name: Sulforaphane

.	Sample name	Description	Rep	Sample Position	Response	IS Response	Response Ratio	Known Conc	Calc Conc	% Dev	Manual
1	7STD1A	STD	1	2:A,12	20670	520075	0.040	5.00	5.81	16.26	None
2	7STD1B	STD	1	2:B,1	11967	390609	0.031	5.00	4.26	-14.79	None
3	7STD2A	STD	1	2:B,2	30399	483252	0.063	10.00	9.76	-2.38	None
4	7STD2B	STD	1	2:B,3	22772	358043	0.064	10.00	9.88	-1.19	None
5	7STD3	STD	1	2:B,4	69986	453099	0.154	25.00	25.37	1.49	None
6	7STD4	STD	1	2:B,5	134961	460153	0.293	50.00	49.05	-1.91	None
7	7STD5	STD	1	2:B,6	264116	431140	0.613	100.00	103.49	3.49	None
8	7STD6	STD	1	2:B,7	652110	440491	1.480	250.00	251.46	0.58	None
9	7STD7	STD	1	2:B,8	1161772	387861	2.995	500.00	509.76	1.95	None
10	7STD8	STD	1	2:B,9	2364141	392244	6.027	1000.00	1026.72	2.67	None
11	7STD9A	STD	1	2:B,10	3684435	321817	11.449	2000.00	1951.15	-2.44	None
12	7STD9B	STD	1	2:B,11	3986166	352884	11.296	2000.00	1925.08	-3.75	None

Figure 1c Standard Curve of SFN in Artificial Cerebrospinal Fluid, P&A Run 3

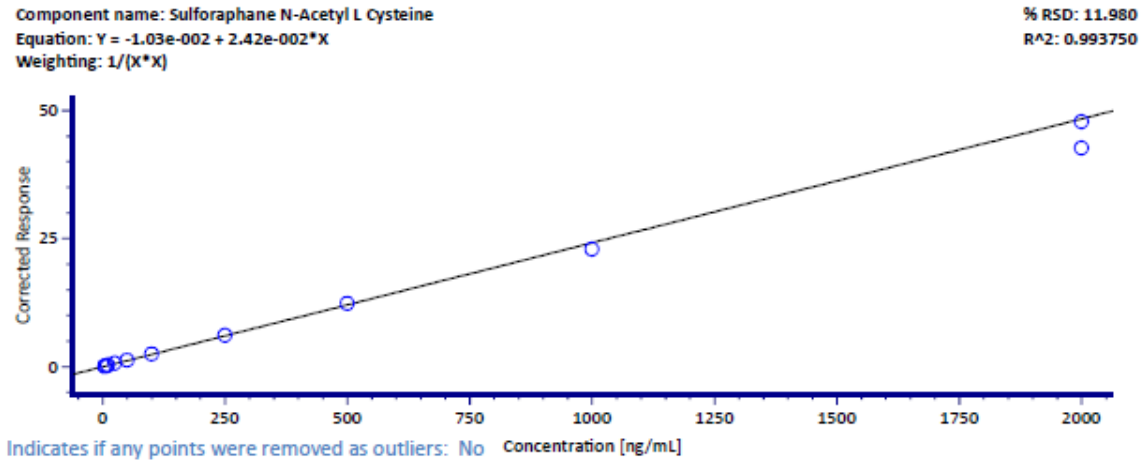


Indicates if any points were removed as outliers: No

Component name: Sulforaphane

.	Sample name	Description	Rep	Sample Position	Response	IS Response	Response Ratio	Known Conc	Calc Conc	% Dev	Manual
1	8STD1A	STD	1	2:A,12	24594	591608	0.042	5.00	5.79	15.73	None
2	8STD1B	STD	1	2:B,1	18886	599493	0.032	5.00	4.14	-17.12	None
3	8STD2A	STD	1	2:B,2	44212	621056	0.071	10.00	10.62	6.19	None
4	8STD2B	STD	1	2:B,3	37455	594365	0.063	10.00	9.29	-7.15	None
5	8STD3	STD	1	2:B,4	98436	586450	0.168	25.00	26.39	5.55	None
6	8STD4	STD	1	2:B,5	183719	555973	0.330	50.00	52.92	5.83	None
7	8STD5	STD	1	2:B,6	375320	583475	0.643	100.00	103.95	3.95	None
8	8STD6	STD	1	2:B,7	787194	510677	1.541	250.00	250.49	0.20	None
9	8STD7	STD	1	2:B,8	1495760	478263	3.127	500.00	509.24	1.85	None
10	8STD8	STD	1	2:B,9	2772807	469504	5.906	1000.00	962.52	-3.75	None
11	8STD9A	STD	1	2:B,10	5058987	429318	11.784	2000.00	1921.49	-3.93	None
12	8STD9B	STD	1	2:B,11	5637221	496099	11.363	2000.00	1852.85	-7.36	None

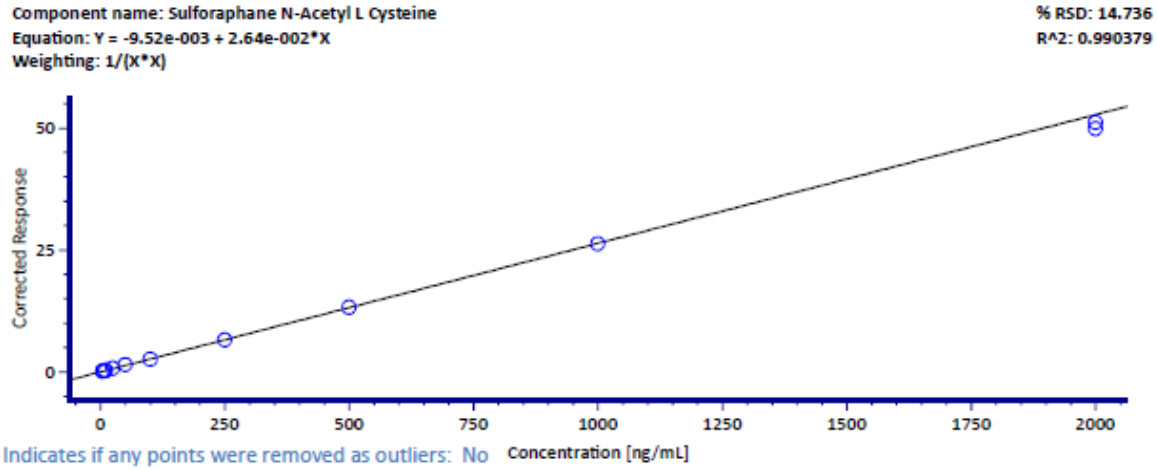
Figure 2a Standard Curve of SFN-NAC in Artificial Cerebrospinal Fluid, P&A Run 1



Component name: Sulforaphane N-Acetyl L Cysteine

.	Sample name	Description	Rep	Sample Position	Response	IS Response	Response Ratio	Known Conc	Calc Conc	% Dev	Manual
1	6STD1A	STD	1	2:A,12	27609	259045	0.107	5.00	4.83	-3.39	None
2	6STD1B	STD	1	2:B,1	26172	246121	0.106	5.00	4.82	-3.60	None
3	6STD2A	STD	1	2:B,2	79642	356124	0.224	10.00	9.67	-3.32	None
4	6STD2B	STD	1	2:B,3	79892	303476	0.263	10.00	11.31	13.06	None
5	6STD3	STD	1	2:B,4	193450	297421	0.650	25.00	27.31	9.23	None
6	6STD4	STD	1	2:B,5	396795	322932	1.229	50.00	51.21	2.42	None
7	6STD5	STD	1	2:B,6	805260	330698	2.435	100.00	101.06	1.06	None
8	6STD6	STD	1	2:B,7	2082294	341905	6.090	250.00	252.13	0.85	None
9	6STD7	STD	1	2:B,8	3901468	316663	12.321	500.00	509.63	1.93	None
10	6STD8	STD	1	2:B,9	8193942	357480	22.921	1000.00	947.76	-5.22	None
11	6STD9A	STD	1	2:B,10	13978974	327794	42.646	2000.00	1762.95	-11.85	None
12	6STD9B	STD	1	2:B,11	14718309	307801	47.818	2000.00	1976.71	-1.16	None

Figure 2b Standard Curve of SFN-NAC in Artificial Cerebrospinal Fluid, P&A Run 2

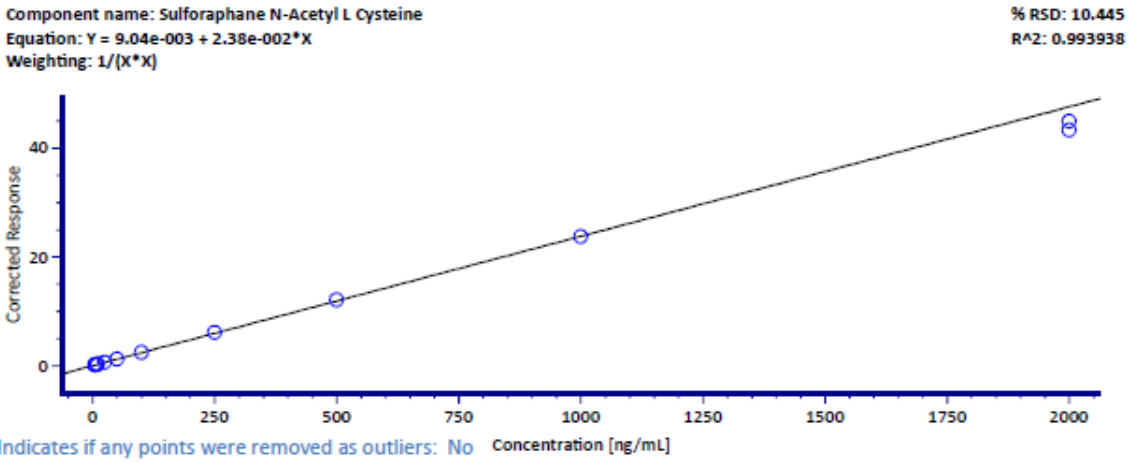


Indicates if any points were removed as outliers: No

Component name: Sulforaphane N-Acetyl L Cysteine

.	Sample name	Description	Rep	Sample Position	Response	IS Response	Response Ratio	Known Conc	Calc Conc	% Dev	Manual
1	7STD1A	STD	1	2:A,12	28833	207963	0.139	5.00	5.61	12.29	None
2	7STD1B	STD	1	2:B,1	31445	292279	0.108	5.00	4.44	-11.25	None
3	7STD2A	STD	1	2:B,2	50999	235953	0.216	10.00	8.55	-14.49	None
4	7STD2B	STD	1	2:B,3	77972	283640	0.275	10.00	10.78	7.77	None
5	7STD3	STD	1	2:B,4	167235	238545	0.701	25.00	26.93	7.70	None
6	7STD4	STD	1	2:B,5	335290	234942	1.427	50.00	54.44	8.88	None
7	7STD5	STD	1	2:B,6	684762	264444	2.589	100.00	98.48	-1.52	None
8	7STD6	STD	1	2:B,7	1932919	294801	6.557	250.00	248.81	-0.47	None
9	7STD7	STD	1	2:B,8	3773403	285654	13.210	500.00	500.92	0.18	None
10	7STD8	STD	1	2:B,9	7436664	283529	26.229	1000.00	994.26	-0.57	None
11	7STD9A	STD	1	2:B,10	12228210	238916	51.182	2000.00	1939.80	-3.01	None
12	7STD9B	STD	1	2:B,11	15286270	306565	49.863	2000.00	1889.82	-5.51	None

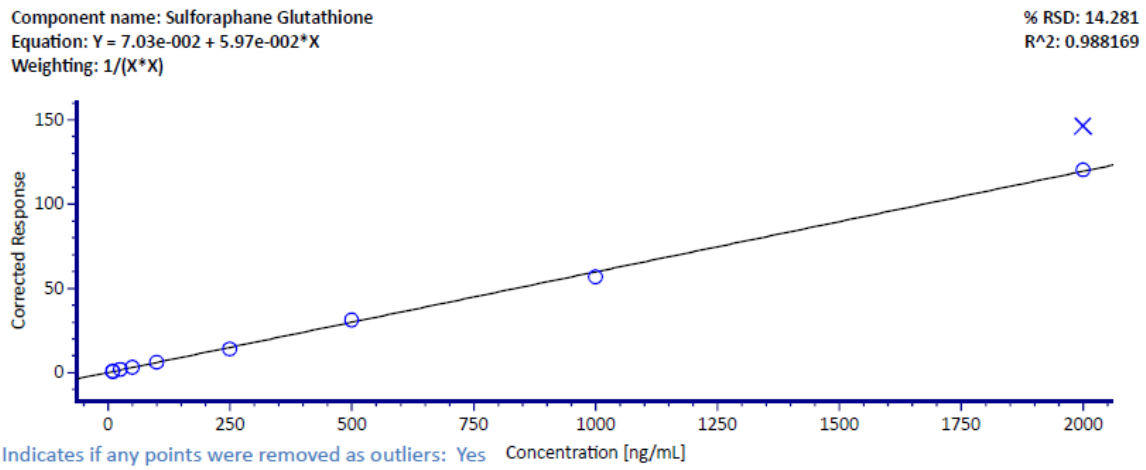
Figure 2c Standard Curve of SFN-NAC in Artificial Cerebrospinal Fluid, P&A Run 3



Component name: Sulforaphane N-Acetyl L Cysteine

.	Sample name	Description	Rep	Sample Position	Response	IS Response	Response Ratio	Known Conc	Calc Conc	% Dev	Manual
1	8STD1A	STD	1	2:A,12	44185	337026	0.131	5.00	5.13	2.53	None
2	8STD1B	STD	1	2:B,1	30494	266869	0.114	5.00	4.42	-11.62	None
3	8STD2A	STD	1	2:B,2	94606	363698	0.260	10.00	10.54	5.45	None
4	8STD2B	STD	1	2:B,3	79144	285511	0.277	10.00	11.26	12.62	None
5	8STD3	STD	1	2:B,4	216669	362456	0.598	25.00	24.73	-1.10	None
6	8STD4	STD	1	2:B,5	391225	321046	1.219	50.00	50.80	1.59	None
7	8STD5	STD	1	2:B,6	905448	371393	2.438	100.00	102.01	2.01	None
8	8STD6	STD	1	2:B,7	2051103	337135	6.084	250.00	255.12	2.05	None
9	8STD7	STD	1	2:B,8	3859667	319402	12.084	500.00	507.11	1.42	None
10	8STD8	STD	1	2:B,9	7290146	307005	23.746	1000.00	996.87	-0.31	None
11	8STD9A	STD	1	2:B,10	12621699	291309	43.328	2000.00	1819.23	-9.04	None
12	8STD9B	STD	1	2:B,11	12680652	282001	44.967	2000.00	1888.06	-5.60	None

Figure 3a Standard Curve of SFN-GSH in Artificial Cerebrospinal Fluid, P&A Run 1

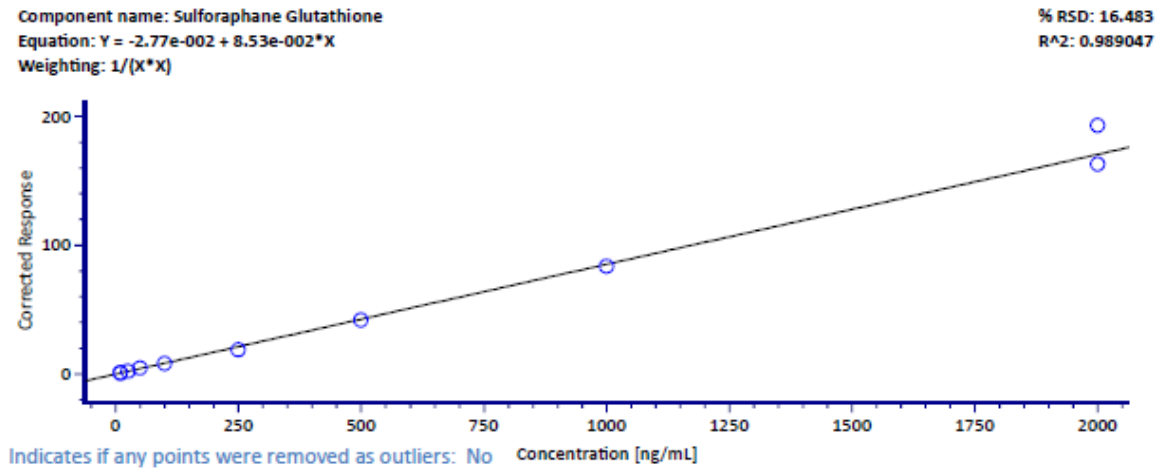


Indicates if any points were removed as outliers: Yes

Component name: Sulforaphane Glutathione

.	Sample name	Description	Rep	Sample Position	Response	IS Response	Response Ratio	Known Conc	Calc Conc	% Dev	Manual
1	6STD1A	STD	1	2:A,12	109347	259045	0.422		5.90		None
2	6STD1B	STD	1	2:B,1	117300	246121	0.477		6.81		None
3	6STD2A	STD	1	2:B,2	202441	356124	0.568	10.00	8.35	-16.51	
4	6STD2B	STD	1	2:B,3	223637	303476	0.737	10.00	11.17	11.72	None
5	6STD3	STD	1	2:B,4	521303	297421	1.753	25.00	28.20	12.78	None
6	6STD4	STD	1	2:B,5	985571	322932	3.052	50.00	49.97	-0.06	None
7	6STD5	STD	1	2:B,6	1983288	330698	5.997	100.00	99.33	-0.67	None
8	6STD6	STD	1	2:B,7	4782094	341905	13.987	250.00	233.22	-6.71	None
9	6STD7	STD	1	2:B,8	9837114	316663	31.065	500.00	519.43	3.89	None
10	6STD8	STD	1	2:B,9	20263915	357480	56.685	1000.00	948.80	-5.12	None
11	6STD9A	STD	1	2:B,10	39411724	327794	120.233	2000.00	2013.79	0.69	None
12	6STD9B	STD	1	2:B,11	45020466	307801	146.265	2000.00	2450.05	22.50	Manual Calibration

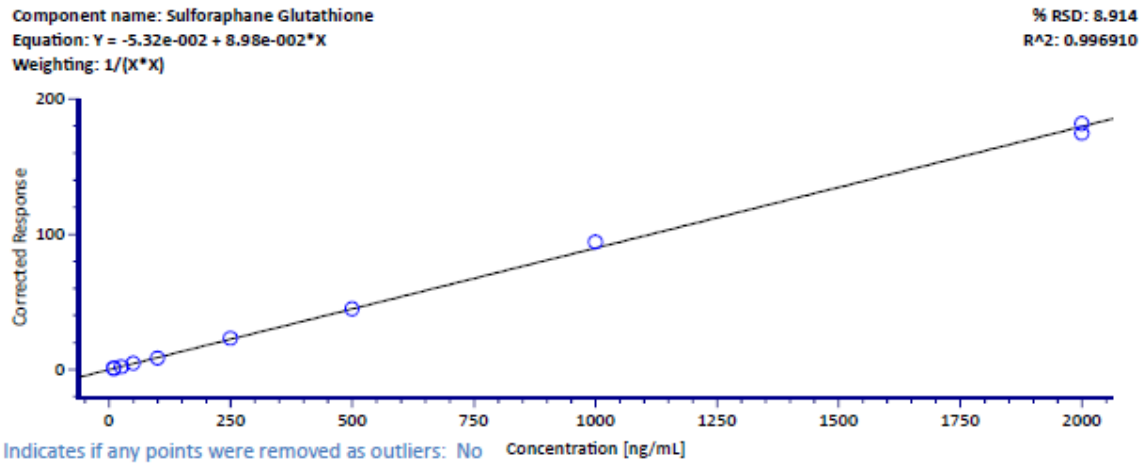
Figure 3b Standard Curve of SFN-GSH in Artificial Cerebrospinal Fluid, P&A Run 2



Component name: Sulforaphane Glutathione

.	Sample name	Description	Rep	Sample Position	Response	IS Response	Response Ratio	Known Conc	Calc Conc	% Dev	Manual
1	7STD1A	STD	1	2:A,12	119443	207963	0.574		7.06		None
2	7STD1B	STD	1	2:B,1	122288	292279	0.418		5.23		None
3	7STD2A	STD	1	2:B,2	214517	235953	0.909	10.00	10.99	9.89	None
4	7STD2B	STD	1	2:B,3	203482	283640	0.717	10.00	8.74	-12.60	None
5	7STD3	STD	1	2:B,4	523173	238545	2.193	25.00	26.05	4.20	None
6	7STD4	STD	1	2:B,5	1091149	234942	4.644	50.00	54.80	9.60	None
7	7STD5	STD	1	2:B,6	2153782	264444	8.145	100.00	95.86	-4.14	None
8	7STD6	STD	1	2:B,7	5539910	294801	18.792	250.00	220.75	-11.70	None
9	7STD7	STD	1	2:B,8	11952416	285654	41.842	500.00	491.11	-1.78	None
10	7STD8	STD	1	2:B,9	23660083	283529	83.449	1000.00	979.13	-2.09	None
11	7STD9A	STD	1	2:B,10	46076118	238916	192.855	2000.00	2262.41	13.12	None
12	7STD9B	STD	1	2:B,11	49906397	306565	162.792	2000.00	1909.79	-4.51	None

Figure 3c Standard Curve of SFN-GSH in Artificial Cerebrospinal Fluid, P&A Run 3



Indicates if any points were removed as outliers: No

Component name: Sulforaphane Glutathione

.	Sample name	Description	Rep	Sample Position	Response	IS Response	Response Ratio	Known Conc	Calc Conc	% Dev	Manual
1	8STD1A	STD	1	2:A,12	165239	337026	0.490		6.05		None
2	8STD1B	STD	1	2:B,1	125195	266869	0.469		5.81		None
3	8STD2A	STD	1	2:B,2	326423	363698	0.898	10.00	10.58	5.82	None
4	8STD2B	STD	1	2:B,3	231155	285511	0.810	10.00	9.60	-3.97	None
5	8STD3	STD	1	2:B,4	749435	362456	2.068	25.00	23.61	-5.57	None
6	8STD4	STD	1	2:B,5	1493808	321046	4.653	50.00	52.38	4.77	None
7	8STD5	STD	1	2:B,6	3070059	371393	8.266	100.00	92.60	-7.40	None
8	8STD6	STD	1	2:B,7	7786101	337135	23.095	250.00	257.66	3.06	None
9	8STD7	STD	1	2:B,8	14327021	319402	44.856	500.00	499.87	-0.03	None
10	8STD8	STD	1	2:B,9	28945464	307005	94.283	1000.00	1050.04	5.00	None
11	8STD9A	STD	1	2:B,10	52917789	291309	181.655	2000.00	2022.55	1.13	None
12	8STD9B	STD	1	2:B,11	49230194	282001	174.575	2000.00	1943.74	-2.81	None

Figure 4 Representative Chromatogram of a Human Cerebrospinal Fluid Blank

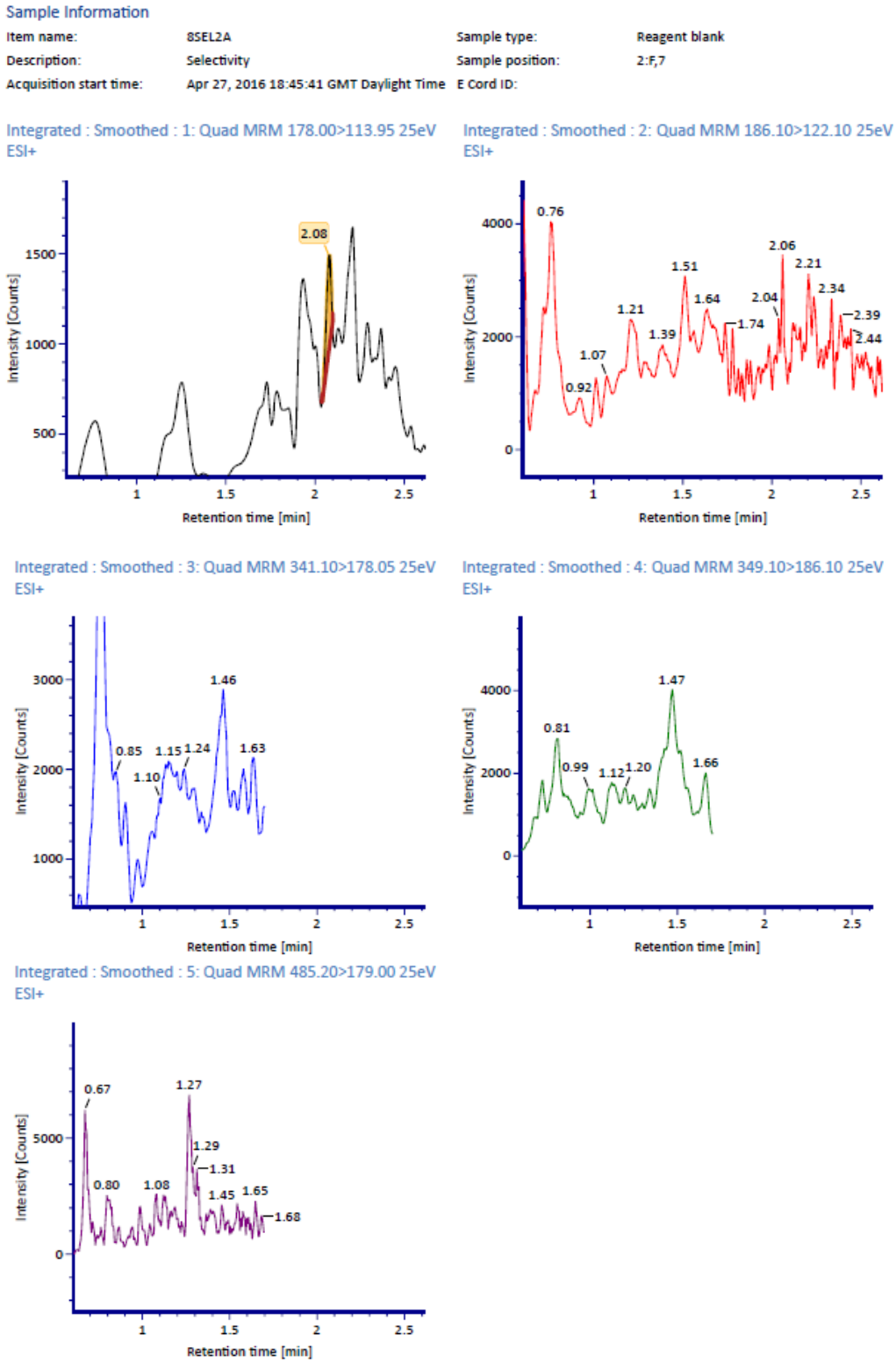


Figure 5 **Representative Chromatogram of an LLOQ Calibration Standard for SFN and SFN-NAC in Artificial Cerebrospinal Fluid**

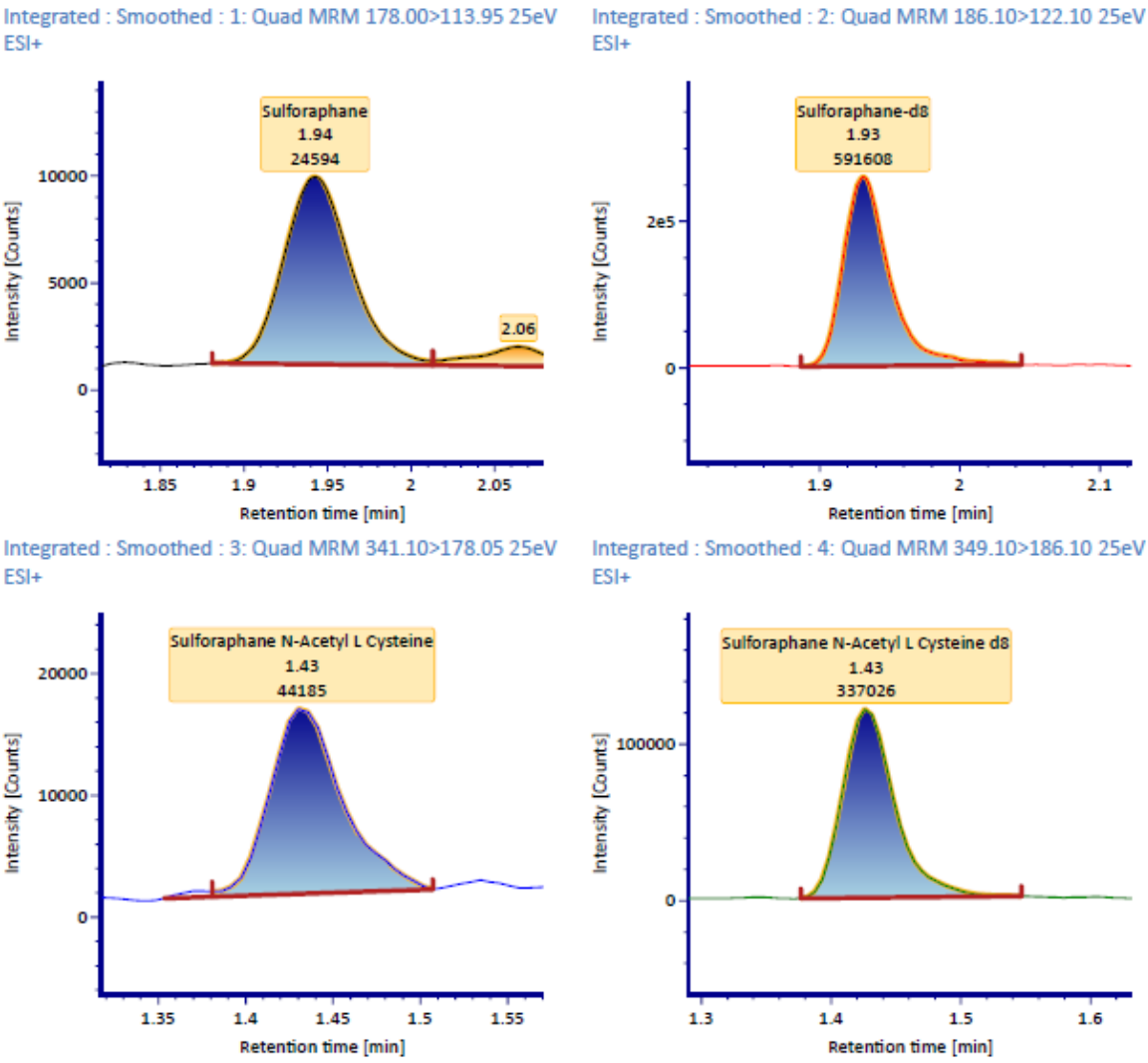


Figure 6 **Representative Chromatogram of an LLOQ Calibration Standard for SFN-GSH in Artificial Cerebrospinal Fluid**

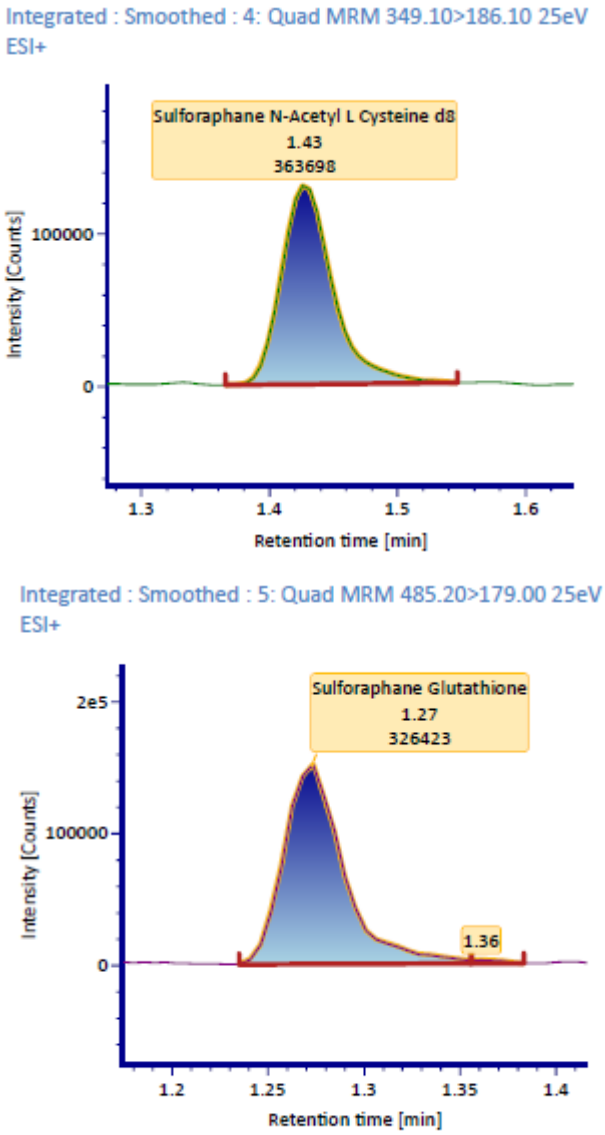
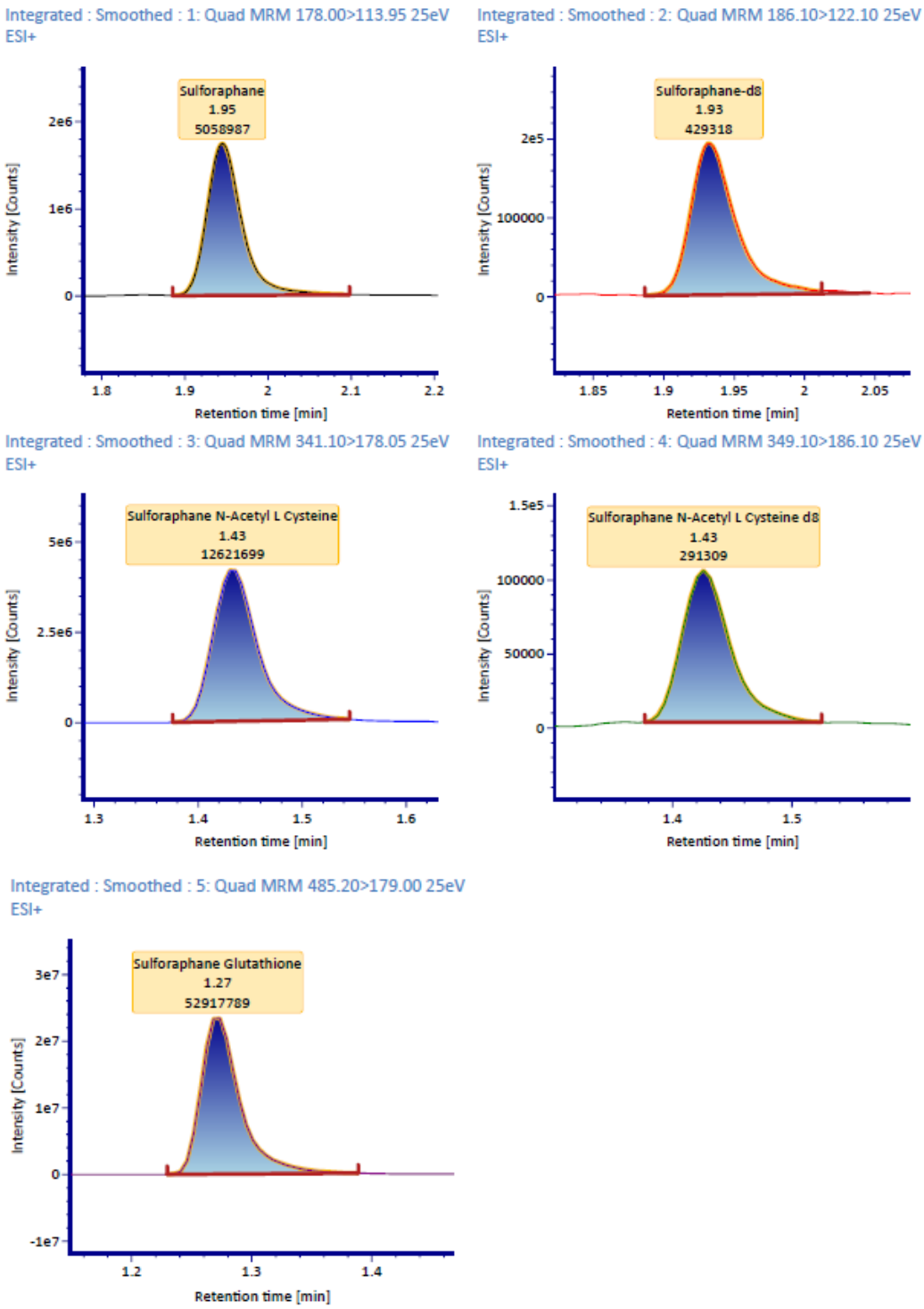


Figure 7 **Representative Chromatogram of a ULOQ Calibration Standard for SFN, SFN-NAC and SFN-GSH in Artificial Cerebrospinal Fluid**



9 AMENDMENT HISTORY

As of the report issue date, no amendments have been issued for this report.

10 APPENDICES

Appendix 1 Bioanalytical Method

Alderley Analytical

METHOD FOR THE DETERMINATION OF D,L SULFORAPHANE, D,L SULFORAPHANE GLUTATHIONE AND D,L SULFORAPHANE N-ACETYL CYSTEINE IN HUMAN CEREBROSPINAL FLUID (CSF) BY LC-MS/MS USING ARTIFICIAL CSF AS A SURROGATE MATRIX

Date	Signed By Reviewer:
25APR2016	

Date	Signed by Approver:
25APR2016	
Justification	I approve this document for content

Date	Signed by Project Leader:
Justification	I approve this method for use with Project No.:

Contents

1 INTRODUCTION

CSF extracts are analysed for D,L Sulforaphane (SFN), D,L Sulforaphane glutathione (SFN-GSH) & D,L Sulforaphane N-acetyl cysteine (SFN-NAC) by LC-MS/MS. D,L Sulforaphane-d8 and D,L-Sulforaphane N-acetyl cysteine-d8 sodium salt are added as internal standards. The calibration range is between 5 and 2000 ng/mL for D,L Sulforaphane and D,L Sulforaphane N-acetyl cysteine, and between 10 and 2000 ng/mL for D,L Sulforaphane glutathione.

Alternative preparations are allowed and must be fully documented.
Equivalent substitutions may be made.
Adjustable pipettes are used in this method.
Unless otherwise specified, all volumetric glassware is Class A.
Weights and volumes may be scaled as needed.
Alternate volumes of solutions and quality control samples may be prepared as needed.
Purity and freebase conversion will vary depending on drug standard lot (refer to certificate of analysis).
Scan functions may differ slightly upon infusion to the instrument.
The suppliers of reagents and matrix can differ from those indicated in the method, but the grade used must be comparable to that stated.

2 MATERIALS AND EQUIPMENT

2.1 Analytical Standards

D,L-Sulforaphane (SFN)

Empirical Formula:	$C_6H_{11}NOS_2$
Molecular Weight:	177.29

D,L-Sulforaphane glutathione (SFN-GSH)

Empirical Formula:	$C_{16}H_{28}N_4O_7S_3$
Molecular Weight:	484.61

D,L-Sulforaphane N-acetyl cysteine (SFN-NAC)

Empirical Formula:	$C_{11}H_{20}N_2O_4S_3$
Molecular Weight:	340.49

D,L-Sulforaphane-d8 (Internal Standard)

Empirical Formula: $C_6H_3D_8NOS_2$

Molecular Weight: 185.34

D,L-Sulforaphane N-acetyl cysteine-d8 Sodium Salt (Internal Standard)

Empirical Formula: $C_{11}H_{11}D_8N_2NaO_4S_3$

Molecular Weight: 370.51

2.2 Reagents and Matrix

Reagent	Grade	Supplier
DMSO	Reagent	Fisher
Acetonitrile	HPLC	Fisher
Ultra-pure water	Milli-Q	In House
Formic acid	98-100%	Sigma Aldrich
Methanol	LC-MS	Fisher
Ammonium acetate	HPLC	Fisher
Sodium chloride	≤99.5%	Sigma Aldrich
Potassium chloride	≤99.0%	Sigma Aldrich
Magnesium chloride hexahydrate	≤99.0%	Sigma Aldrich
Sodium phosphate dibasic dihydrate	≤99.0%	Sigma Aldrich
Calcium chloride dihydrate	AR	Fisher
Sodium phosphate monobasic monohydrate	≤98%	Sigma Aldrich
Citric acid	99.5-100.5 %	Sigma Aldrich
Matrix	**Product Number**	**Supplier**
Human CSF	N/A	Supplied by Sponsor

2.3 Consumables

Description	Supplier	Product Number
ACE 5 C18-AR 50 x 3.0 mm C18 Column	Hichrom Ltd.	ACE-129-0503
Oasis HLB µElution Plate 30 µm	Waters	186001828BA

3 REAGENT PREPARATION

3.1 Mobile Phase A - 0.01% Aqueous Formic Acid

100 µL of formic acid is added to 1000 mL Milli-Q ultra-pure water. The mobile phase is sonicated for ~10 minutes after preparation.

3.2 Mobile Phase B - Acetonitrile

1000 mL of HPLC grade acetonitrile is transferred to a 1 litre bottle. The mobile phase is sonicated for ~10 minutes after preparation.

3.3 Sample Manager Wash

900 mL of HPLC grade acetonitrile and 100 mL of Milli-Q ultra-pure water are measured out separately and mixed together. The sample manager wash is sonicated for ~10 minutes after preparation.

3.4 Seal/Purge Wash

100 mL of HPLC grade acetonitrile and 900 mL of Milli-Q ultra-pure water are measured out separately and mixed together. The seal/purge wash is sonicated for ~10 minutes after preparation.

3.5 Purge Wash Solution (for infusion)

350 mL of HPLC grade acetonitrile and 150 mL of Milli-Q ultra-pure water are measured out separately and mixed together. The purge wash solution is sonicated for ~10 minutes after preparation.

3.6 10 mM Ammonium Acetate Solution

0.76 g of HPLC grade ammonium acetate is weighed out and transferred to a 1000 mL volumetric flask and dissolved in 1 litre of Milli-Q ultra-pure water.

3.7 10 mM Ammonium Acetate/MeCN (50:50)

50 mL of MeCN and 50 mL of 10 mM Ammonium Acetate Solution are measured out separately and mixed together.

3.8 0.5M Citric Acid

9.6 g of citric acid is weighed out and is dissolved in 100 mL of Milli-Q ultra-pure water.

3.9 Solution A for Artificial CSF

8.66 g of sodium chloride, 0.224 g of potassium chloride, 0.206 g of calcium chloride dihydrate and 0.163 g of magnesium chloride hexahydrate are weighed out and dissolved in 500 mL of Milli-Q ultra-pure water.

3.10 Solution B for Artificial CSF

0.027 g of sodium phosphate monobasic monohydrate and 0.142 g of sodium phosphate dibasic dihydrate are weighed out and dissolved in 500 mL of Milli-Q ultra-pure water.

3.11 Artificial CSF

50 mL of Solution A for Artificial CSF is mixed with 50 mL of Solution B for Artificial CSF.

3.12 Stabilised CSF

2 mL of 0.5 M citric acid is added to 100 mL blank human CSF or blank artificial CSF.

4 D,L SULFORAPHANE, D,L SULFORAPHANE GLUTATHIONE AND D,L SULFORAPHANE N-ACETYL CYSTEINE SOLUTION PREPARATION FOR CALIBRATION STANDARDS

Prepare a stock solution of D,L Sulforaphane by accurately weighing approximately 1 mg of D,L Sulforaphane into an amber glass vial and accurately adding the correct volume of DMSO to make a 1 mg/mL solution, taking into account any correction factor for purity. Mix well. Solution is labelled as "D,L Sulforaphane Solution A".

Prepare a stock solution of D,L Sulforaphane glutathione by accurately weighing approximately 1 mg of D,L Sulforaphane glutathione into an amber glass vial and accurately adding the correct volume of methanol to make a 1 mg/mL solution, taking into account any correction factor for purity. Mix well. Solution is labelled as "D,L Sulforaphane glutathione Solution B".

Prepare a stock solution of D,L Sulforaphane N-acetyl cysteine by accurately weighing approximately 1 mg of D,L Sulforaphane N-acetyl cysteine into an amber glass vial and accurately adding the correct volume of methanol to make a 1 mg/mL solution, taking into account any correction factor for purity. Mix well. Solution is labelled as "D,L Sulforaphane N-acetyl cysteine Solution C".

Prepare "Solution D" in a polypropylene bottle by accurately measuring 200 µL of "D,L Sulforaphane Solution A", 200 µl of "D,L Sulforaphane glutathione Solution B" and 200 µL of "D,L Sulforaphane N-acetyl cysteine Solution C", and mixing with 4400 µL of 10 mM Ammonium Acetate/MeCN (50:50) to make a 40 µg/mL solution of all three analytes.

5 CALIBRATION STANDARD PREPARATION

Prepare a set of calibration standards for D,L Sulforaphane, D,L Sulforaphane glutathione & D,L Sulforaphane N-acetyl cysteine in blank stabilised artificial CSF in micro centrifuge tubes at the following concentrations, as shown in Table 1.

Table 1: Calibration Standards

Solution ID	Solution Used	Volume (µL)	Stabilised aCSF (µL)	Overall Volume (mL)	Calibration Standard Concentration (ng/mL)
STD9	D (40 µg/mL)	50	950	1	2000
STD8	STD9	500	500	1	1000
STD7	STD8	500	500	1	500
STD6	STD7	500	500	1	250
STD5	STD6	500	750	1.25	100
STD4	STD5	500	500	1	50
STD3	STD4	500	500	1	25
STD2	STD3	500	750	1.25	10
STD1	STD2	500	500	1	5

NB – STD1 is not used for D,L Sulforaphane glutathione analysis.

Calibration standards should be mixed well and prepared daily. After preparation, calibration standards should be kept on wet ice before extraction.

6 D,L SULFORAPHANE, D,L SULFORAPHANE GLUTATHIONE AND D,L SULFORAPHANE N-ACETYL CYSTEINE SOLUTION PREPARATION FOR QUALITY CONTROL SAMPLES

Prepare a stock solution of D,L Sulforaphane by accurately weighing approximately 1 mg of D,L Sulforaphane into an amber glass vial and accurately adding the correct volume of DMSO to make a 1 mg/mL solution, taking into account any correction factor for purity. Mix well. Solution is labelled as "D,L Sulforaphane Solution E".

Prepare a stock solution of D,L Sulforaphane glutathione by accurately weighing approximately 1 mg of D,L Sulforaphane glutathione into an amber glass vial and accurately adding the correct volume of methanol to make a 1 mg/mL solution, taking into account any correction factor for purity. Mix well. Solution is labelled as "D,L Sulforaphane glutathione Solution F".

Prepare a stock solution of D,L Sulforaphane N-acetyl cysteine by accurately weighing approximately 1 mg of D,L Sulforaphane N-acetyl cysteine into an amber glass vial and accurately adding the correct volume of methanol to make a 1 mg/mL solution, taking into account any correction factor for purity. Mix well. Solution is labelled as "D,L Sulforaphane N-acetyl cysteine Solution G".

Prepare "Solution H" in a polypropylene bottle by accurately measuring 200 µL of "D,L Sulforaphane Solution E", "200 µL of "D,L Sulforaphane glutathione Solution F" and 200 µL of "D,L Sulforaphane N-acetyl cysteine Solution G", and mixing with 4400 µL of 10 mM Ammonium Acetate/MeCN (50:50) to make a 40 µg/mL solution of all three analytes.

7 BULK QUALITY CONTROL SAMPLES

Prepare a set of bulk QC samples for D,L Sulforaphane, D,L Sulforaphane glutathione & D,L Sulforaphane N-acetyl cysteine in blank stabilised artificial CSF in micro centrifuge tubes as shown in Table 2.

Table 2: Quality Control Samples

Solution ID	Solution Used	Volume (µL)	Stabilised aCSF (µL)	Overall Volume (mL)	QC Sample Concentration (ng/mL)
QCDIL	H (40 µg/mL)	800	3200	4	8000
QC5	H (40 µg/mL)	500	9500	10	2000
QC4	QC5	8000	2000	10	1600
QC3	QC4	2500	2500	5	800
QC2	QC3	225	11775	12	15
QC1	QC2	2000	4000	6	5
GSHQC2	QC3	450	11550	12	30
GSHQC1	GSHQC2	2000	4000	6	10

NB – GSHQC2 and GSHQC1 are used for D,L Sulforaphane glutathione analysis only.

Bulk Quality Control Samples should be mixed well, frozen at -80°C and defrosted on wet ice prior to use. Appropriate volumes for sets of each individual quality control sample will be pipetted out prior to freezing.

The sample volume for each quality control sample will be 700 µL.

8 INTERNAL STANDARDS

Mix all preparations well before use. Store preparations in polypropylene bottles at nominal 4°C.

1 mg/mL D,L Sulforaphane-d8

D,L Sulforaphane-d8 is supplied as an exact weight of approximately 1 mg. Add the correct volume of DMSO to make a 1 mg/mL solution. Mix well. Solution is labelled as "Internal Standard Solution V".

1 mg/mL D,L Sulforaphane N-acetyl cysteine-d8 sodium salt

D,L Sulforaphane N-acetyl cysteine-d8 sodium salt is supplied as an exact weight of approximately 1 mg. Add the correct volume of DMSO to make a 1 mg/mL solution. Mix well. Solution is labelled as "Internal Standard Solution W".

10 µg/mL D,L Sulforaphane-d8 & D,L Sulforaphane N-acetyl cysteine-d8 sodium salt

Prepare "Internal Standard Solution X" by accurately measuring 50 µL of Internal Standard Solution V and 50 µL of Internal Standard Solution W, and mixing with 4900 µL of 10 mM Ammonium Acetate/MeCN (50:50).

100 ng/mL D,L Sulforaphane-d8 & D,L Sulforaphane-d8 N-Acetyl-L-cysteine sodium salt

Prepare "Internal Standard Solution Y" by accurately measuring 200 µL of "Internal Standard Solution X" and mixing with 19800 µL of 10 mM Ammonium Acetate/MeCN (50:50).

Addition of 50 µL of "Internal Standard Solution Y" to the sample volume of 100 µL gives an in-sample IS concentration of 50 ng/mL for D,L Sulforaphane-d8 and 50 ng/mL for D,L Sulforaphane-d8 N-acetyl-L-cysteine sodium salt.

9 PRE-EXTRACTION PROCEDURE

Before beginning the extraction procedure, ensure the following samples have been prepared and/or are available:

1.	100 µL of appropriate STD samples have been aliquoted into a 2mL 96 well collection plate.
2.	Prepare a blank standard (STD0) by adding 100 µL of blank stabilised aCSF to a 2mL 96 well collection plate.
3.	100 µL of appropriate QC samples have been aliquoted into a 2mL 96 well collection plate.
4.	Study samples are available and thawed, and 100 µL of each study sample aliquoted into a 2mL 96 well collection plate.

5.	Enough CSF blanks have been prepared, by adding 100 µL blank stabilised aCSF to a 2mL 96 well collection plate.

10 EXTRACTION PROCEDURE

1.	Add 50 µL of Internal Standard Solution Y to each calibration standard (including STD0), quality control samples and study samples, **but not CSF blanks**.
2.	Add 50 µL of 10 mM Ammonium Acetate/MeCN (50:50) to the plasma blank samples **only**. All samples should now be 150 µL in volume, made up of 100 µL aCSF (or human CSF) and 50 µL of 10 mM Ammonium Acetate/MeCN (50:50).
3.	Add 300 µL of 0.5M citric acid solution to each sample, and mix using a 1200 µL multipipette.
4.	Ensure the waste plate is in place. Condition the HLB µElution plate with 200 µL of methanol. Apply a vacuum of ~ -13 in. Hg until the methanol has pulled through the plate.
5.	Repeat step 4 with 200 µL of MilliQ ultra-pure water.
6.	Remove the waste collection plate and insert the load collection plate. Load the standards, blanks, and study samples onto the HLB µElution plate using a 1200 µL multipipette. Once all samples are loaded, quickly switch the vacuum on then off, and allow the analytes to interact with the SPE bed for approximately 5 minutes. Then apply a vacuum of ~ -13 in. Hg until the run has pulled through the plate.
7.	Remove the load collection plate and reinsert the waste plate. Wash with 200 µL of MilliQ ultra-pure water. Apply a vacuum of ~ -13 in. Hg until the water has pulled through the plate.
8.	Remove waste plate and insert the elution collection plate.
9.	Elute with 2 x 50 µL of acetonitrile.
10.	Remove the elution collection plate from SPE device and transfer 100 µL of the load collection samples into the elution collection plate ensuring that samples are transferred in the same well plate conformation.
11.	Apply heat seal.

11 SYSTEM SUITABILITY

The system suitability will be verified before each sample analysis batch run by performing a system suitability test. The system suitability test will cover the following areas:

- Carryover - An extracted sample at a concentration equivalent to the ULOQ will be injected, followed by an injection of a blank. Any response in the blank at the retention time of the analytes must be less than 20% of the subsequent LLOQ sample response.

Any response in the blank at the retention time of the internal standards must be less than 5% of the internal standard response in the LLOQ sample.

- Absolute Retention - The absolute retention time must be within the range determined during the method qualification.
- Sensitivity - An extracted sample at the LLOQ will be injected directly after a blank sample and must show a signal-to-noise ratio equal to or greater than 5:1 to be acceptable.
- Chromatography - Peak shape will be assessed based on scientific experience of the Laboratory Scientist and/or the Project Leader.

12 LC-MS/MS METHOD

The LC-MS/MS method is attached as a pdf file (see Appendix 1). Peak processing settings may be optimised on a batch by batch basis.

13 AMENDMENT HISTORY

Method	Version	Changes from previous version
0001/023	V01	First version; Effective Date 30 Mar 2016
0001/023	V02	Version 02; change to calibration standard preparation, change of preparation to IS solution Y, minor change to extraction procedure; Effective Date 08 Apr 2016
0001/023	V03	Version 03; change to the preparation of stabilised aCSF, changes to the extraction procedure. Effective Date 25Apr2016

Appendix 2 Certificates of Analysis

CERTIFICATE OF ANALYSIS

Toronto Research Chemicals Inc.
2 Brisbane Road, Toronto, ON, M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

1. Identification

CAS Number:
4478-93-7

Catalogue Number:
S699115

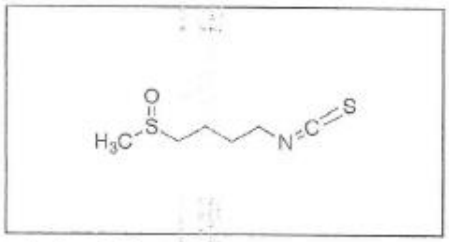
LGC Standards GmbH
Mercatorstr. 51
46485 Wesel
Germany

Tel: +49 (0)281 9887 0
Fax: +49 (0)281 9887 199
Email: de@lgcstandards.com
Web: www.lgcstandards.com

Product:
D,L-Sulforaphane

Synonyms:
1-Isothiocyanato-4-(methylsulfinyl)-butane; 4-Methylsulfinylbutyl Isothiocyanate; Sulforaphan;

Structure:



Molecular Formula:
$C_6H_{11}NOS_2$

Molecular Weight:
177.29

Source of Product:
Synthetic

2. Analytical Information

Lot Number:
5-YMK-40-1

Melting Point:
N/A

Boiling Point:
N/A

Atmosphere:
Inert Gas

Appearance of Product:
Pale Yellow Oil

Solubility
Chloroform (Slightly), Ethyl Acetate (Slightly)

Method for Determining Identity:
^{1}H NMR (DMSO-d_6, $CDCl_3$), ^{13}C NMR (DMSO-d_6), FT-IR, and MS

Stability
Light Sensitive

Purity:
95%

Long Term Storage Condition:
Amber Vial, -20°C Freezer, Under Inert Atmosphere

Additional Information:
TLC Conditions: SiO_2; Dichloromethane : Methanol = 7 : 1; Visualized with UV and AMCS; Single Spot, Rf = 0.45.
^{1}H NMR, ^{13}C NMR, FT-IR, and MS conform to structure.
Elemental Analysis: (Found) %C: 39.12, %H: 6.90, %N: 7.52; (Calculated) %C: 40.65, %H: 6.25, %N: 7.90

Philip Chan, Head of Quality Assurance

QC Test Date
October 7, 2015

Retest Date
October 5, 2018

Toronto Research Chemicals Inc.
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CERTIFICATE OF ANALYSIS

2 Brisbane Road, Toronto, ON. M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

1. Identification

CAS Number:
836682-32-7

Catalogue Number:
S699117

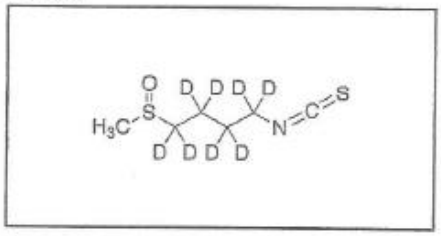
LGC Standards GmbH
Mercatorstr. 51
46485 Wesel
Germany

Tel: +49 (0)281 9887 0
Fax: +49 (0)281 9887 199
Email: de@lgcstandards.com
Web: www.lgcstandards.com

Product:
D,L-Sulforaphane-d8

Synonyme:
1-Isothiocyanato-4-(methylsulfinyl)-butane-d8; 4-Methylsulfinylbutyl Isothiocyanate-d8; Sulforaphan-d8;

Structure:



Molecular Formula:
$C_6H_3D_8NOS_2$

Molecular Weight:
185.34

Source of Product:
Synthetic

2. Analytical Information

Lot Number:
10-SBT-79-5

Melting Point:
N/A

Boiling Point:
N/A

Atmosphere:
Inert Gas

Appearance of Product:
Orange - Yellow Oil

Solubility
Chloroform (Slightly), Methanol (Slightly)

Method for Determining Identity:
^{1}H NMR ($CDCl_3$) and MS

Stability
Hygroscopic

Purity:
Chemical Purity: 98%
Isotopic Purity: 99.7%

Long Term Storage Condition:
Hygroscopic, Refrigerator, Under Inert Atmosphere

Additional Information:
TLC Conditions: SiO_2; Dichloromethane : Methanol = 9 : 1; Visualized with UV and $KMnO_4$; Rf = 0.75.
^{1}H NMR and MS conform to structure.
Elemental Analysis: (Found) %C: 38.96, %H: 6.05, %N: 7.13; (Calculated) %C: 38.88, %H: 5.99, %N: 7.56
Normalized Intensities: d_0 = 0.02%, d_1 = 0.00%, d_2 = 0.01%, d_3 = 0.00%, d_4 = 0.00%, d_5 = 0.08%, d_6 = 0.04%, d_7 = 1.56%, d_8 = 98.29%

Philip Chan, Head of Quality Assurance

QC Test Date
July 28, 2015

Retest Date
July 26, 2020

CERTIFICATE OF ANALYSIS

Toronto Research Chemicals Inc.
2 Brisbane Road, Toronto, ON. M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

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1. Identification

CAS Number:
334829-66-2

Catalogue Number:
S699120

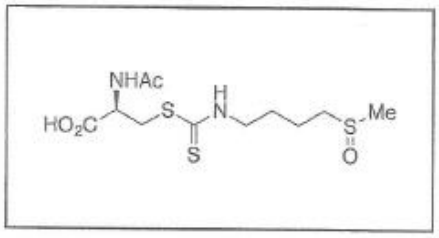
LGC Standards GmbH
Mercatorstr. 51
46485 Wesel
Germany

Tel: +49 (0)281 9887 0
Fax: +49 (0)281 9887 199
Email: de@lgcstandards.com
Web: www.lgcstandards.com

Product:
D,L-Sulforaphane N-Acetyl-L-cysteine

Synonyms:
N-Acetyl-S-[[[4-(methylsulfinyl)butyl]amino]thioxomethyl]-L-cysteine; Sulforaphane NAC;

Structure:



Molecular Formula:
$C_{11}H_{20}N_2O_4S_3$

Molecular Weight:
340.49

Source of Product:
N/A

2. Analytical Information

Lot Number:
6-GHZ-12-1

Melting Point:
58 - 63°C

Boiling Point:
N/A

Atmosphere:
Inert Gas

Appearance of Product:
Off-White Solid

Solubility
Methanol (Slightly)

Method for Determining Identity:
NMR (CD_3OD) and MS

Stability
Hygroscopic

Purity:
98%

Long Term Storage Condition:
Hygroscopic, -20°C Freezer, Under Inert Atmosphere

Additional Information:
TLC Conditions: C_{18}; Acetonitrile : Water = 9 : 1; Visualized with UV and AMCS; Single Spot, Rf = 0.50.
^1H NMR and MS conform to structure.
Specific Rotation: +11.1° (c = 0.5, Water)

Philip Chan, Head of Quality Assurance

QC Test Date
September 24, 2015

Retest Date
September 22, 2020

CERTIFICATE OF ANALYSIS

Toronto Research Chemicals Inc.
2 Brisbane Road, Toronto, ON, M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

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1. Identification

CAS Number:
N/A

Catalogue Number:
S699122

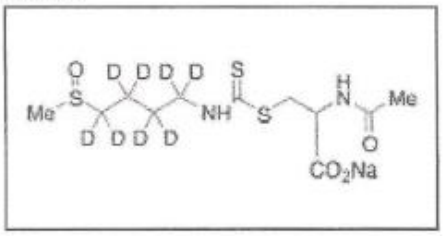
LGC Standards GmbH
Mercatorstr. 51
46485 Wesel
Germany

Tel: +49 (0)281 9887 0
Fax: +49 (0)281 9887 199
Email: de@lgcstandards.com
Web: www.lgcstandards.com

Product:
D,L-Sulforaphane-d8 N-Acetyl-L-cysteine Sodium Salt

Synonyms:
N-Acetyl-S-[[[4-(methylsulfinyl)butyl-d8]amino]thioxomethyl]-L-cysteine Sodium Salt; Sulforaphane NAC Sodium Salt;

Structure:



Molecular Formula:
$C_{11}H_{11}D_8N_2NaO_4S_3$

Molecular Weight:
370.51

Source of Product:
Synthetic

2. Analytical Information

Lot Number:
1-YSS-36-3

Melting Point:
>165.0°C (dec.)

Boiling Point:
N/A

Atmosphere:
Inert Gas

Appearance of Product:
Off-White Solid

Solubility
Methanol, Water

Method for Determining Identity:
^{1}H NMR (CD_3OD) and MS

Stability
Hygroscopic

Purity:
Chemical Purity: 95%
Isotopic Purity: 99.6%

Long Term Storage Condition:
Hygroscopic, -20°C Freezer, Under inert atmosphere

Additional Information:
TLC Conditions: C_{18}; Acetonitrile : Water = 9 : 1; Visualized with UV and $KMnO_4$; Single Spot, Rf = 0.45.
^{1}H NMR and MS conform to structure.
Elemental Analysis: (Found) %C: 33.28, %H: 5.72, %N: 7.01; (Calculated) %C: 37.91, %H: 5.79, %N: 8.04
Normalized Intensity: d_0 = 0.00%, d_1 = 0.00%, d_2 = 0.00%, d_3 = 0.00%, d_4 = 0.00%, d_5 = 0.00%, d_6 = 0.34%, d_7 = 2.68%, d_8 = 96.98%.
Water Content: 12.5% by Karl Fischer
Sodium Content: 5.67%

Philip Chan, Head of Quality Assurance

QC Test Date
August 14, 2014

Retest Date
August 12, 2017

CERTIFICATE OF ANALYSIS

Toronto Research Chemicals Inc.
2 Brisbane Road, Toronto, ON. M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

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1. Identification

CAS Number:
289711-21-3

Catalogue Number:
S699430

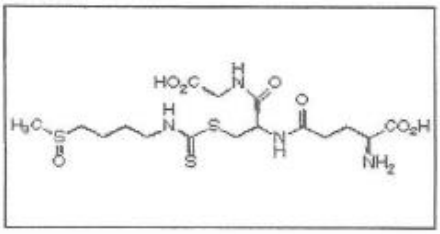
LGC Standards GmbH
Mercatorstr. 51
46485 Wesel
Germany

Tel: +49 (0)281 9887 0
Fax: +49 (0)281 9887 199
Email: de@lgcstandards.com
Web: www.lgcstandards.com

Product:
D,L-Sulforaphane Glutathione

Synonyms:
L-γ-Glutamyl-S-[[[4-(methylsulfinyl)butyl]amino]thioxomethyl]-L-cysteinylglycine; SFN-GSH;

Structure:



Molecular Formula:
$C_{15}H_{28}N_4O_7S_3$

Molecular Weight:
484.61

Source of Product:
Synthetic

2. Analytical Information

Lot Number:
1-CRD-3-1

Melting Point:
147 - 153°C (dec.)

Boiling Point:
N/A

Atmosphere:
Inert Gas

Appearance of Product:
Off-White Solid

Solubility
Methanol (Sparingly), Water (Sparingly)

Method for Determining Identity:
¹H NMR (D_2O) and MS

Stability
Hygroscopic

Purity:
95%

Long Term Storage Condition:
Hygroscopic, -20°C Freezer, Under inert atmosphere

Additional Information:
TLC Conditions: C_{18}; Methanol : Water = 9 : 1; Visualized with UV, AMCS, and Ninhydrin; Rf = 0.70.
¹H NMR and MS conform to structure.

Philip Chan, Head of Quality Assurance

QC Test Date
December 24, 2013

Retest Date
December 22, 2018

Appendix 3 Validation Plan

Bioanalytical Validation Plan Amendment 1 for the Determination of Sulforaphane (SFN), SFN N-acetyl Cysteine and SFN Glutathione in Human Cerebrospinal Fluid (CSF) by LC-MS/MS, using Artificial CSF as a Surrogate Matrix

Additions are indicted in bold italics text. Deletions are indicated by a strikethrough of the text.

Reasons for amendment:

1. Change to assigned Bioanalytical Project Leader due to extended absence.
2. Analyte previously named SFN N-Glutathione in error; changed in all places to SFN Glutathione.
3. An increase in the LLOQ for SFN Glutathione from 5 ng/mL to 10 ng/mL. The increase to the LLOQ is required for SFN Glutathione due to results obtained during method development for the selectivity of the assay for SFN Glutathione. When using an LLOQ of 5 ng/mL the blank samples analysed for the selectivity assessment were found to have SFN Glutathione peak areas that were greater than 20% of the LLOQ peak area. As a result the LLOQ will be increased.
4. Due to point 3 the calibration range for SFN Glutathione will be amended appropriately.
5. Correction of typographical errors.

Study Sponsor	Evgen Ltd. 146 Brownlow Hill Liverpool L3 5RF
Sponsor Contact	Dr David Howat d.howat@evgen.com Office +44 (0)151 705 3532 Cell +44 (0)7341 479346
Bioanalytical Test Site	Alderley Analytical BioHub at Alderley Park Alderley Edge Cheshire SK10 4TG
Bioanalytical Project Leader	~~Claire Wildgoose~~ ~~claire.wildgoose@alderleyanalytical.com~~ ~~Office +44 (0)1625 238610~~ ***Alan Gibbs*** ***alan.gibbs@alderleyanalytical.com*** ***Office +44 (0)1625 238610***
Bioanalytical Method Title	Method for the Determination of SFN, SFN N-acetyl Cysteine and SFN Glutathione in CSF by LC-MS/MS
Alderley Analytical Method Number	0001/023
Alderley Analytical Study Number	0014/003

Species/Matrix/Stabiliser	Human / CSF / 0.5M citric acid
Stabiliser Concentration	***300 µL of 0.5M citric acid per 17.7 mL of CSF***
Surrogate Matrix	Artificial stabilised CSF
Projected Experimental Start Date*	~~March 2016~~ ***April 2016***
Projected Experimental Completion Date*	~~May 2016~~ ***June 2016***
Reference Standards	SFN SFN N-acetyl Cysteine SFN Glutathione SFN-d8 (Internal Standard for SFN) SFN-d8 N-acetyl Cysteine-d8 sodium salt (Internal Standard for SFN N-acetyl Cysteine and SFN Glutathione)

* A change in date does not necessitate an amendment to this document.

SIGNATURES OF APPROVAL

I hereby approve this validation study plan amendment and agree that this study will be conducted in accordance with the validation study plan and amendment, applicable regulatory requirements and the Alderley Analytical Standard Operating Procedures.

Alan Gibbs
Project Leader

23 May 2016
Date

I hereby assign the above-named Project Leader to the validation study described in this validation study plan amendment. The above named Project Leader is a permanent replacement for the previously assigned Project Leader.

Elizabeth Thomas
Elizabeth Thomas
CEO

23 May 2016
Date

I hereby approve this validation study plan amendment.

Dave Chadwick
David Chadwick
Study Sponsor Representative

23 MAY 2016
Date

TABLE OF CONTENTS

1 INTRODUCTION

The validation procedures outlined in this validation plan follow Standard Operating Procedure (SOP) L001 (Ref. 1), which is broadly based on the Guidance for Industry document titled: "Bioanalytical Method Validation" generated by the U.S. Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), May 2001 and the European Medicines Agency "Guideline on Bioanalytical Method Validation", effective from 1 February 2012.

1.1 Objective

The full validation of a method for the quantitative analysis of SFN, SFN N-acetyl Cysteine and SFN Glutathione in human CSF with a calibration range of 5 to 2000 ng/mL ***for SFN and SFN N-acetyl Cysteine and 10 to 2000 ng/mL for SFN Glutathione***, using artificial CSF as a surrogate matrix.

1.2 Compliance

This study will be conducted in a laboratory inspected by the United Kingdom Good Laboratory Practice Monitoring Authority (GLPMA), and in accordance with Medicines and Healthcare products Regulatory Agency (MHRA) published guidance on Good Laboratory Practice for Clinical Laboratories. All work performed at Alderley Analytical will be carried out in accordance with the Alderley Analytical Standard Operating Procedures.

No formal claim of compliance will be made for this validation study.

1.3 Quality Assurance Evaluation

The Alderley Analytical Quality Assurance Unit will be responsible for the following quality assurance functions on the study:

- Validation Plan review
- Study specific procedure audits
- Data/report audit

2 METHOD

2.1 Analysis Method Summary

An LC-MS/MS (Liquid Chromatography with Tandem Mass Spectrometry) assay has been developed for the measurement of SFN, SFN N-acetyl Cysteine and SFN Glutathione in human stabilised CSF, using artificial stabilised CSF as a surrogate matrix. The method involves solid phase extraction from 100 µL of stabilised CSF. Separation is achieved using an ACE 5 C18-AR analytical column and a mobile phase consisting of 0.01% aqueous formic acid and acetonitrile.

2.2 Equipment

The equipment to be used for the determination of SFN, SFN N-acetyl Cysteine and SFN Glutathione in human and artificial CSF comprises of a Waters Acquity I-Class UPLC coupled to a Waters Xevo TQ-S Mass Spectrometer.

LC-MS/MS operation and data acquisition will be performed using UNIFI software V1.7.

2.3 Surrogate Matrix

The artificial CSF consists of the following components:

- Sodium chloride (NaCl)
- Potassium chloride (KCl)
- Calcium chloride dihydrate ($CaCl_2 . 2H_2O$)
- Magnesium chloride hexahydrate ($MgCl . 6H_2O$)
- Sodium dihydrogen phosphate (NaH_2PO_4)
- Disodium hydrogen phosphate dihydrate ($Na_2HPO_4 . 2H_2O$)

The preparation of the artificial CSF will be fully documented in the study file.

The artificial CSF will be stabilised with *300* µL of 0.5M citric acid per *17.7* mL of artificial CSF.

2.4 Preparation of Standard Calibration Curves

Calibration standards are prepared by adding diluted standard solutions of SFN in dimethyl sulfoxide (DMSO) and SFN N-acetyl Cysteine and SFN Glutathione in methanol to blank artificial stabilised CSF to create calibration standards at concentrations of 5.0 (Lower Limit of Quantification (LLOQ)), 10, 25, 50, 100, 250, 500, 1000 and 2000 (Upper Limit of Quantification (ULOQ)) ng/mL **for SFN and SFN N-acetyl Cysteine and 10 (Lower Limit of Quantification (LLOQ)), 25, 50, 100, 250, 500, 1000 and 2000 (Upper Limit of Quantification (ULOQ)) ng/mL for SFN Glutathione**. Single replicates of each calibration standard are produced, other than for those at the LLOQ and ULOQ, which are prepared in duplicate. Calibration curves will be produced by the simplest and most appropriate linear regression mode to describe the concentration-response relationship.

2.5 Preparation of Quality Control (QC) Samples

Bulk QC samples are prepared in artificial stabilised CSF and stored at a nominal temperature of -80°C at the start of method validation. QCs are prepared by adding diluted standard solutions of SFN in DMSO and SFN N-acetyl Cysteine and SFN Glutathione in methanol to artificial stabilised CSF to give a range of concentrations of approximately 5, 15, 800, 1600 and 2000 ng/mL **for *SFN and SFN N-acetyl Cysteine and 10, 30, 800, 1600 and 2000 ng/mL for SFN Glutathione*.**

The QC samples should be prepared from different weighings of the analytical reference standards from those used to prepare the calibration curve. Alternatively, the QC samples may be prepared from

the same weighings as the standards, if the weighings in solution compare within ±5% to another independent weighing according to the formula below.

% difference = [(M1 – M2)/average of M1 and M2)] * 100

The concentrations of SFN, SFN N-acetyl Cysteine and SFN Glutathione in the QC samples are calculated by reference to the appropriate calibration curve and then compared to the theoretical (nominal) concentrations.

QC samples stored at a nominal -80°C will be analysed in a minimum of three validation batch runs, other than the QC sample at the ULOQ, which is analysed in one run only.

A dilution QC is prepared in replicates of 6 at 8000 ng/mL, see section 3.3.

3 FULL VALIDATION

3.1 Linear Range and Response Function

The relationship between response and concentration should be demonstrated to be continuous and reproducible. **A minimum of** eight levels of non–zero standards, as described in section 2.4, are used for constructing a calibration curve. In addition, a blank (named "Reagent Blank") and a "zero" standard (blank with internal standard, named "Blank") are analysed. One replicate of each standard is injected in ascending order at the start of the batch run. The remaining ULOQ and LLOQ standards should be run at the end of the batch, with all other samples in the batch run 'bracketed' by these two sets of calibration standards. Duplicate blank samples should also be injected after each ULOQ standard (see section 3.8).

Typically, a weighted linear regression is used, though the simplest relationship providing the best accuracy for the back-calculated concentrations for the standards is employed. For multiple analyte assays, the simplest weighting may differ for one or more analyte. In such cases, the more complex weighting may be used for all analytes in the method.

3.1.1 Acceptance Criteria for Each Calibration Curve

Any calibration standard having an assignable cause for rejection will be removed from the calibration curve first. Any remaining standards not meeting the acceptance criteria i.e. having a back-calculated concentration with a deviation of more than ±15% (±20% at the LLOQ) from its nominal concentration are then excluded from the calibration curve and the curve is sequentially reprocessed, beginning with the standard having the greatest percent difference from nominal. This procedure is repeated until all remaining standards are acceptable.

To accept a calibration curve, for each analyte:

- The standard curve must be constructed from a minimum of 75% of all of the calibration standards analysed in a run (including any that are excluded for assignable cause).
- At least one replicate each at the LLOQ and ULOQ must be included.

- The standard curve must contain at least one calibration standard at each of a minimum of six concentration levels.

3.2 Accuracy, Precision (Reproducibility), Sensitivity (LLOQ), and Upper Limit of Quantification (ULOQ)

Intra-run and inter-run accuracy and precision are determined by analysing artificial stabilised CSF quality control (QC) samples, as described in section 2.5, in replicates of at least six, at a minimum of four concentrations (LLOQ, low, mid and high), over a minimum of three batches on at least two different days.

During at least one batch run, extra samples at the ULOQ concentration are prepared in replicates of six (not including the standards used in the calibration curve) to assess accuracy and precision at the upper curve limit (see section 3.2.2).

3.2.1 Acceptance Criteria for the Accuracy and Precision (including the LLOQ) of the Method

For each validation occasion the intra-run accuracy and precision of the method will be determined at each concentration level, for each analyte as follows:

- For each concentration level, intra-run precision is defined as the coefficient of variation (CV) at each concentration level for each individual run. Precision should not exceed 15%, except at the LLOQ where a 20% limit is acceptable.
- For each concentration level, intra-run accuracy is defined as the percent bias (% bias) at each concentration level for each individual run. Acceptable limits are ±15% of the nominal concentration, except at the LLOQ where a ±20% limit is acceptable.
- For the LLOQ QC samples all results are used to calculate accuracy and precision, regardless of whether the observed concentration is below the nominal LLOQ concentration.

Note: If two consecutive validation occasions fail to meet the above intra-run acceptance criteria, the method will be re-evaluated and re-initiated when any issues have been resolved.

Across all validation occasions the accuracy and precision of the method will be determined at each concentration level as follows:

- Inter-run precision, defined as the CV, is calculated from all QCs analysed on all precision and accuracy validation occasions, at each concentration level. Precision should not exceed 15%, except at the LLOQ where a 20% limit is acceptable.
- Inter-run accuracy, defined as the %bias, is calculated from all QCs analysed on all precision and accuracy validation occasions at each concentration level. Acceptable limits are ±15% of the nominal concentration, except at the LLOQ where a ±20% limit is acceptable.

Quality control samples may be excluded from the tabulated data only if:

- There is assignable cause (e.g. anomalous chromatography or lack of internal standard).
- The sample is lost during the extraction or analysis.

Outliers can also be excluded if proven significant by a statistical method. Within a run, no more than a single replicate at each concentration level may be rejected as an outlier for calculation of precision and accuracy. If outliers are excluded, results calculated both with and without outliers will be presented in the validation report.

3.2.2 Acceptance Criteria for the Accuracy and Precision of the Upper Limit of Quantitation (ULOQ) of the Method

All results for the ULOQ samples should be used to calculate accuracy and precision, regardless of whether the observed concentration is above the nominal ULOQ concentration.

- Precision (coefficient of variation (CV)) for the six replicates should not exceed 15%.
- Accuracy for the six replicates should not deviate from the nominal concentration by more than ±15%.

3.2.3 Sensitivity of the Method

The LLOQ QC samples from all precision and accuracy batch runs are evaluated for adequate sensitivity, and must ideally demonstrate a signal to noise ratio of ≥5:1. Signal to noise of 5:1 is not mandatory and ultimately the definitive evidence of assay performance is that the precision and accuracy calculated at the LLOQ is within the acceptable limits of precision and accuracy.

3.3 Dilution Integrity

A QC sample (QC Dil) will be prepared in human stabilised CSF at a concentration of 8000 ng/mL for SFN, SFN N-acetyl Cysteine and SFN Glutathione, and diluted into the calibration range using a dilution factor of 10. The QC Dil sample is frozen for at least 24 hours, thawed, diluted, extracted, and analysed in replicates of at least six. The dilution of the QC Dil sample will be performed using artificial stabilised CSF. The replicates of the QC Dil sample must have a mean accuracy (%bias) of within ±15% of the nominal concentration, and the precision (%CV) must not exceed 15%, to be acceptable.

3.4 Recovery (Extraction Efficiency)

Recovery is determined by analysing at least three replicates of extracted samples at the low, mid, and high QC sample concentrations, along with at least three replicates of fortified samples at concentrations equivalent to extracted low, mid, and high QC sample concentrations. The recovery of the internal standards are determined in a similar manner at the working concentrations of the internal standards.

No formal acceptance limits are placed on the minimal recovery required; however, recovery should be consistent over the calibration range of the assay. The precision of recovery samples at each concentration level should not exceed 15%.

3.5 Selectivity

Six lots of blank matrix are processed and analysed in single replicates according to the method being validated. The six lots will be made up of the following;

- 1 pooled human CSF sample
- 3 individual human CSF donors
- 1 individual human CSF donor containing a level of blood (estimated to be approximately 0.1%)
- 1 lot of artificial CSF

The blank matrix samples should not produce any significant interference (i.e., a response (peak area, height or appropriate ratio)) that is:

- Greater than 20% of the average lower limit of quantification (LLOQ) response at the retention times of SFN, SFN N-acetyl Cysteine and SFN Glutathione.
- Greater than 5% of the mean internal standard response at the retention times of the internal standards.

However, in the event that run response deteriorates or improves over the course of the batch run, it is acceptable to compare the blank matrix to the LLOQ standard that was injected closest to the matrix blank.

If one of the lots fails to meet the above acceptance criteria, then the number of lots should be increased to 10, and at least 90% of the lots should not produce any significant interference. If two or more lots fail to meet the above acceptance criteria, the method should be investigated prior to proceeding.

3.6 Matrix Effects for LC-MS/MS Methods

Six lots of blank matrix (as described in section 3.5) are extracted in triplicate and then fortified with SFN, SFN N-acetyl Cysteine, SFN Glutathione and internal standards at a concentration equivalent to an extracted low QC sample and at a concentration equivalent to an extracted high QC sample (a total of 36 fortified samples (18 at each concentration level)). Additionally, two analytical solutions containing SFN, SFN N-acetyl Cysteine, SFN Glutathione and internal standards are prepared in triplicate in reconstitution solution, also at concentrations equivalent to an extracted low QC sample and an extracted high QC sample (a total of 6 analytical samples (3 at each level)). These are analysed in the following order:

Fortified lot 1 replicate 1, fortified lot 2 replicate 1, etc., with the three analytical solutions dispersed among the fortified samples (low QC level samples), followed by the same injection order for all high QC level samples.

The matrix effect is calculated for each lot using the Matrix Factor equation below:

$$\text{Matrix Factor} = \frac{\text{Mean Peak Area in Presence of Matrix Ions}}{\text{Mean Peak Area in Absence of Matrix Ions}}$$

At each concentration assessed, the overall precision of the matrix factors across all six lots should be ≤15%. If the overall precision of the matrix factors is not within these limits, the assessment may still

be considered acceptable if the overall precision of the normalised (IS adjusted) matrix factors across all six lots is ≤15%.

The normalised matrix effect is calculated for each lot using the equation below:

$$\text{Normalised Matrix Factor} = \frac{\text{Mean Peak Area Ratio in Presence of Matrix Ions}}{\text{Mean Peak Area Ratio in Absence of Matrix Ions}}$$

If both the Matrix Factor and Normalised Matrix Factor fail to meet the above acceptance criteria, then the method should be re-evaluated. However, precision outside the above acceptance criteria may be accepted, but the rationale for acceptance must be fully documented in the study file.

3.6.1 Evaluation of Human Stabilised CSF

The matrix effect of human stabilised CSF on the precision and accuracy of the method will be assessed. QC samples at low and high concentrations are prepared by spiking SFN, SFN N-acetyl Cysteine and SFN Glutathione into human stabilised CSF.

Six replicates of each QC sample, stored at the requisite temperature (nominal -80ºC) for at least 24 hours prior to use, will be analysed in a minimum of one validation batch run, along with a standard curve and run acceptance QCs in at least duplicate, prepared in artificial stabilised CSF.

Acceptance criteria for the human stabilised CSF QCs are the same as the criteria defined in section 3.2.1 for intra-run precision and accuracy.

3.6.2 Evaluation of CSF containing blood

The matrix effect resulting from the presence of blood in a CSF sample on the precision and accuracy of the method will be assessed. QC samples at low and high concentrations are prepared by spiking SFN, SFN N-acetyl Cysteine and SFN Glutathione into artificial stabilised CSF containing 0.1% K_2EDTA whole blood.

Six replicates of each QC sample, stored at the requisite temperature (nominal -80ºC) for at least 24 hours prior to use, will be analysed in a minimum of one validation batch run, along with a standard curve and run acceptance QCs in at least duplicate, prepared in artificial stabilised CSF.

Acceptance criteria for the QCs containing blood are the same as the criteria defined in section 3.2.1 for intra-run precision and accuracy.

3.7 Evaluation of a Large Run Size

At least one batch run should be injected that is approximately equivalent in size to a prospective sample analysis run. QCs should be extracted in replicates of at least six, but LLOQ QC samples need not be tested as part of this assessment.

The desired batch size may be achieved by extracting and injecting extra zero standards (recommended), or if sample volume permits, by re-injecting standards and/or QCs (or an entire batch) as many times as needed to achieve the desired run size. If multiple re-injections of the same QC

samples are made to achieve the desired run size, it must be specified in advance which QC injections are used to quantify this assessment.

For the assessment to be deemed acceptable the calibration curve acceptance criteria stated in section 3.1.1 and the intra-run accuracy and precision acceptance criteria stated in section 3.2.1 must be met. Furthermore, the run should be assessed for any significant bias from the beginning to the end of the run. If bias is observed it should be addressed in the raw data on a run by run basis.

3.8 Carryover Evaluation

Instrument carryover (cross-contamination) is evaluated by analysing two blank samples following each high standard during each validation run. The blanks should not produce any significant carryover i.e. a response (peak area or height) that is greater than 20% of the average LLOQ response, at the retention time of SFN, SFN N-acetyl Cysteine and SFN Glutathione. In addition, the blanks should not produce any significant carryover i.e. a response (peak area or height) that is greater than 5% of the mean internal standard response, at the retention time of the internal standard.

3.9 Stability

For all stability experiments, the date and time of sample removal and return to storage are documented appropriately.

3.9.1 Solution Stability

The solution stability of SFN, SFN N-acetyl Cysteine and SFN Glutathione will be determined during method validation study 0014/001 (Ref 2).

The results obtained will be included as an Appendix in the final report for this method validation.

The stability assessed during method 0014/001 will be as follows;

- Storage under the relevant conditions (i.e. appropriate solvent, container material and storage condition/temperature) for specific time period for the highest concentration and the lowest concentration solutions that are to be stored.
- Solution stability evaluated at ambient temperature after storage for approximately six hours or longer. An aliquot of the solution to be evaluated will be stored at ambient temperature, while another aliquot of the same solution is maintained at the requisite (cold) storage temperature. After storage, the two aliquots are compared.

3.9.2 Extracted Sample Storage

Three assessments are performed to demonstrate extracted sample storage stability and these are typically run concurrently, but may be run individually if needed.

- Extract Stability

- Re-injection Reproducibility
- Autosampler Stability

Extract storage times are established provided that:

- The calibration curve meets criteria in Section 3.1.1.
- At least two-thirds of the stored QC samples at each level (low, medium, and high) are within ±15% of their nominal concentrations.
- The mean concentration of the stored QC samples at each level (low, medium, and high) does not deviate from the nominal concentration by more than ±15%.

Note that the LLOQ QC samples are not used for these assessments.

3.9.2.1 Extract Stability

Extract Stability is determined over the anticipated time that an entire run may be stored prior to analysis. It will be established by storing an entire run (at a minimum; blanks, calibration standards and low, mid and high QC samples in triplicate) for the desired time at the requisite temperature prior to injection. Extract stability is calculated from the completion of sample preparation until injection of the first QC sample or calibration standard.

3.9.2.2 Re-injection Reproducibility / Autosampler Stability

Re-injection reproducibility / autosampler stability will be established by re-analysing a previously injected run (at a minimum, blanks, standards, and triplicate low, medium and high QC samples) after storage at the requisite autosampler temperature for the desired time. Freshly prepared calibration standards must ***not*** be added for the second injection of an entire run.

The re-injected QC samples will be compared separately to both the original calibration curve and to the re-injected calibration curve.

If the complete re-injection run meets acceptance criteria, an entire run may be re-injected during sample analysis after an interruption in analysis.

If the re-injected QCs meet acceptance criteria against the original calibration curve a partial run may be re-injected during sample analysis.

If the re-injected QCs and/or re-injected standards fail to meet acceptance criteria no reinjections may be performed and complete re-extraction and re-analysis will be required during sample analysis.

The re-injection reproducibility / autosampler stability storage time will be calculated from the original injection of the first QC sample or calibration standard until re-injection of the first QC sample or calibration standard.

It must be documented as to whether samples are stored with pierced or re-capped vials after the initial injection.

3.9.3 Matrix Stability

Quality control samples used for stability are typically prepared in bulk, aliquoted to smaller volumes, and stored frozen prior to use. For the following matrix stability assessments, the stability QC samples are extracted along with blanks, and a freshly prepared calibration curve (i.e., prepared fresh from stable stock and/or spiking solutions).

Run acceptance QCs (low, mid, and high concentrations prepared in artificial stabilised CSF, in at least duplicate) must also be analysed if the stability assessment is not conducted in a precision and accuracy run during validation. Run acceptance QC samples are separate from the stability QC samples and must be within proven stability.

At least 2/3 of the run acceptance QC samples, with at least 50% at each level, must be within ±15% of their nominal concentrations.

If degradation is indicated for any of the matrix stability assessments, a shorter storage time may be analysed to determine when the instability occurs. Alternatively, a colder temperature (e.g., an ice bath or colder freezer temperature) or other means of stabilising a sample can be used, and the test repeated.

3.9.3.1 Bench Top Stability at the Required Temperature

Three levels of QC samples (low, high and QC Dil) in both human and artificial stabilised CSF are analysed in at least triplicate after maintaining them at the required temperature for a minimum of four hours prior to extraction. The temperature and storage time assessed must be documented in the study file.

Stability is indicated provided that at least 2/3 of the stability QC samples at each level are within ±15% of their nominal concentrations, and the mean concentration at each level does not deviate from the nominal concentration by more than ±15% in both artificial and human CSF.

3.9.3.2 Freeze/Thaw Stability

Three levels of QC samples (low, high and QC Dil) in both human and artificial stabilised CSF are frozen for at least 24 hours at a nominal temperature of -80ºC and thawed on wet ice. When completely thawed, the samples are then returned to the freezer for at least 12 hours under the same storage conditions. This cycle is performed a minimum of three times. After the final cycle, the samples are analysed in at least triplicate. The acceptance criteria is as above in section 3.9.3.1.

3.9.3.3 Long Term Storage Stability (Frozen Stability)

A separate study, to investigate frozen stability over three time points, has been agreed. This will therefore be covered in a separate analysis plan (study number 0014/004).

3.10 Interference Screens

SFN, SFN N-acetyl Cysteine and SFN Glutathione will be analysed independently to monitor the contribution of each analyte on the other. This will be performed at the LLOQ and ULOQ level for each analyte.

Independent samples containing each separate analyte will be prepared at the lower and upper limit of quantification (without addition of internal standard) in artificial CSF and analysed in triplicate. These will be analysed for the other analytes (i.e. samples spiked with SFN will be analysed for SFN N-acetyl Cysteine and SFN Glutathione, and so on).

The pure standards of the metabolites are known to contain an amount of SFN and therefore rather than assign definitive acceptance criteria for the response observed in the interference samples (typically when spiked at the ULOQ any interference response noted should be less than 20% of the average LLOQ response for the analyte in question), the impact of any interference observed over the concentration range will be assessed and the results discussed in the validation report.

3.11 Retention Time Variability

Absolute retention time is monitored during each validation run by comparing the retention times of analytes and internal standards at the beginning of a run to those at the end of a run. These should vary by no more than 10%.

4 REPORT FORMAT

A validation report will be prepared from the Alderley Analytical validation report template.

4.1 Review of a Validation Prior to Validation Report Release

It is acceptable to begin study sample analysis after the validation is completed but prior to the validation report being reviewed by the Quality Assurance Unit. In such instances, sample analysis may proceed provided that the Project Leader has reviewed the validation data, validation acceptance criteria are met, and the Sponsor is in agreement with this action.

4.2 Issue of the Validation Report

A draft report will be prepared following completion of the study and will be finalised following consultation with the Sponsor and Quality Assurance review. The report will include all information necessary to provide a complete and accurate description of the experimental methods and results and any circumstances that may have affected the quality or integrity of the study.

The Sponsor will receive an electronic version of the draft validation report in a Microsoft Word format. Any comments made by the Sponsor must be returned as tracked changes using the Microsoft Word version. The final validation report will be provided in Adobe Acrobat PDF format. The validation report will be created from electronic files to the extent possible, including text and tables generated by Alderley Analytical. Report entries not available as electronic files and/or original signature pages will

be scanned and converted to PDF files for incorporation into the report. An original copy of the report with Alderley Analytical handwritten signatures will be retained in the Alderley Analytical Archive.

5 ARCHIVE PROCEDURE

All records of the study including the Validation Plan, raw data and approved final report are archived in the Alderley Analytical Archive, as documented according to Alderley Analytical SOP QA009 (Ref. 3). Materials will be retained for a period of two years from the date of finalising the report after which time the Sponsor will be contacted to determine requirements for further storage, return or destruction of materials. No materials will be destroyed without written instruction from the Sponsor.

6 REFERENCES

1. Alderley Analytical SOP L001: Validation of Bioanalytical Methods
2. Alderley Analytical Study Number 0014/001: Validation for the Determination of SFN, SFN N-acetyl Cysteine and SFN Glutathione in Human K_2 EDTA Stabilised Plasma by LC-MS/MS.
3. Alderley Analytical SOP QA009: Archiving Procedures / Records Management

Appendix 4 Stock Stability

Table 15a SFN Stock Stability (Low Conc Solution) over 29 Days

Solution Concentration 40 µg/mL	Stored Soln – 29 Days in 50/50 10 mM Ammonium Acetate/ACN at 4ºC	Fresh Soln
	Peak Area	Peak Area
	648775	636855
	704539	636372
	683944	632412
Mean	679086	635213
SD	28198	2438
CV	4.15%	0.38%
Stability	107%	

Table 15b SFN Stock Stability (High Conc Solution) over 67 Days

Solution Concentration 1 mg/mL	Stored Soln – 67 Days in DMSO at 4ºC	Fresh Soln
	Peak Area	Peak Area
	3360129	3342288
	3325825	3335828
	3305668	3282253
	3257128	3479061
	3292992	3504633
	3278510	3540893
	3531944	3158282
	3559979	3176226
	3602142	3189603
Mean	3390480	3334341
SD	134936	146770
CV	3.98%	4.40%
Stability	102%	

Table 15c SFN-NAC Stock Stability (Low Conc Solution) over 29 Days

Solution Concentration 40 µg/mL	Stored Soln – 29 Days in 50/50 10 mM Ammonium Acetate/ACN at 4°C	Fresh Soln
	Peak Area	Peak Area
	2277997	2185857
	2449085	2183819
	2434323	2156971
Mean	2387135	2175549
SD	94804	16121
CV	3.97%	0.74%
Stability	110%	

Table 15d SFN-NAC Stock Stability (High Conc Solution) over 69 Days

Solution Concentration 1 mg/mL	Stored Soln – 69 Days in Methanol at -20°C	Fresh Soln
	Peak Area	Peak Area
	1073071	1408127
	1076520	1345766
	1049102	1316492
	1160270	1194793
	1132882	1102941
	1160013	1136124
	1237470	1094431
	1236991	1102936
	1248821	1113150
Mean	1152793	1201640
SD	76576	122346
CV	6.64%	10.18%
Stability	96%	

Table 15e SFN-GSH Stock Stability (Low Conc Solution) over 29 Days

Solution Concentration 40 µg/mL	Stored Soln – 29 Days in 50/50 10mM Ammonium Acetate/ACN at 4°C	Fresh Soln
	Peak Area	Peak Area
	10345831	11350731
	11005958	11208759
	10926766	11368572
Mean	10759519	11309354
SD	360445	87573
CV	3.35%	0.77%
Stability	95%	

Table 15f SFN-GSH Stock Stability (High Conc Solution) over 67 Days

Solution Concentration 1 mg/mL	Stored Soln – 67 Days in Methanol at -20°C	Fresh Soln
	Peak Area	Peak Area
	1657416	1307866
	1600269	1400582
	1628381	1434932
	1660799	1507275
	1668402	1517363
	1649089	1485711
	1715400	1534809
	1627455	1512937
	1665618	1553216
Mean	1652536	1472743
SD	32448	78355
CV	1.96%	5.32%
Stability	112%	

Evgen Pharma Plc

Protocol: EVG001SAH
Eudract No.: 2014-003284-38

Statistical Analysis Plan:

A Phase II Safety, Tolerability, Pharmacokinetic and Pharmacodynamic Study of SFX-01 in Subarachnoid Haemorrhage, with exploratory efficacy evaluations. The study is a randomised, double-blind, parallel-group design comparing SFX-01 (300 mg) taken orally as capsules or as a suspension via a nasogastric tube (NG) twice-daily for up to 28 days versus placebo in 90 patients who have had SAH and present within 48 hours of ictus.

Sponsor:	Evgen Pharma plc Liverpool Science Park IC2 146 Brownlow Hill Liverpool L3 5RF
Author & Qualifications	Andrew Stone M.Sc. C.Stat.
Document Date:	04 February 2019
Version/Status:	Final

AUTHORISATION

Position	Name	Signature	Date (dd mon yyyy)
Medical Advisor Evgen Pharma plc	Dr. Thomas Morris	DocuSigned by: Thomas Morris 62C1C1B7C7114D3...	2/4/2019
Clinical Development Officer Evgen Pharma plc	Sally Ross	DocuSigned by: Sally Ross 6A6F318FC77041D...	04 Feb 2019
Project Manager TCTC Ltd	Ingrid Gerber	DocuSigned by: Ingrid Gerber 3E763580DFD443E...	2/5/2019
Statistical Consultant Stone Biostatistics Ltd	Andrew Stone	DocuSigned by: BF0293A0587A44B...	2/4/2019

DOCUMENT HISTORY

Version	Date	Author	Section/Page Amendment
Final	04 Feb 2019	Andrew Stone	Finalised SAP

Table of Contents

List of Abbreviations and Definition Of Terms

AE	Adverse Event
ANOVA	Analysis Of Variance
ATC	Anatomical Therapeutic Chemical
AUC	Area Under the Curve
bid	Two times daily
BICRO-39	Brain Injury Community Rehabilitation Outcomes Scale
BP	Pain
CLCE-24	Checklist for Cognitive and Emotional Consequences CRF
CRF	Case Report Form
CRP	C-reactive protein
CSF	Cerebrospinal Fluid
CSR	Clinical Study Report
CT	Computed Tomography
CV	Coefficient of Variation
DCI	Delayed Cerebral Ischaemia
DSA	Digital Subtraction Angiography
DSMB	Data Safety Monitoring Board
EOS	End of Study
EVD	External Ventricular Drain
EW	Emotional Well-Being
GCS	Glasgow Coma Scale
GH	General Health
GOSE	Glasgow Outcome Scale (Extended)
HL	Hodges-Lehmann
HP	Haptoglobin
ICH	International Conference on Harmonisation
ITT	Intention-to-Treat
IV	Intravenous
LP	Lumbar Puncture
MCA	Middle Cerebral Artery
MDA	Malondialdehyde
MedDRA	Medical Dictionary for Regulatory Activities
MFV	Mean Flow Velocity
MH	Mental Health
MMRM	Mixed Model Repeated Measures
MRA	Magnetic Resonance Angiography
MRI	Magnetic Resonance Imaging
mRS	Modified Rankin Scale
NA	Not Applicable
NG	NasoGastric
PF	Physical Functioning
PK	Pharmacokinetic
PP	Per-Protocol
PTAE	Pre-Treatment Adverse Event
RE	Role Limitations Due to Emotional Problems
RP	Role Limitations Due to Physical Health
SAE	Serious Adverse Event
SAH	Subarachnoid Haemorrhage
SAP	Statistical Analysis Plan
SAHOT	Subarachnoid Haemorrhage Outcome Tool

SF	Social Functioning
SF-36	Short Form (36) Health Survey
SFN	Sulforaphane
SFX-01	The Investigational Medicinal Product/stabilised Sulforaphane
SOC	System Organ Class
TCD	Trans-Cranial Doppler
TCTC	The Clinical Trial Company Ltd
TEAE	Treatment Emergent Adverse Event
VT	Energy/Fatigue
WBC	White blood cells
WFNS	World Federation of Neurosurgical Scale
WHODD	World Health Organization Drug Dictionary
Z	Standardized Normal Distribution Test Statistic

1 Introduction

This document describes the objectives, analysis populations, endpoint derivations, statistical analyses and data presentations to be performed for the clinical protocol EVG001SAH entitled “SFX-01 After Subarachnoid Haemorrhage (SAS Study)”. This is the final version of the SAP text, approved in February 2019 which updates previous draft versions of the SAP to provide more detail on endpoint derivations and the analyses to be performed. In general, details of the study design that can also be found in the protocol have been removed from this version. The supporting tables, listing and figures will be finalised in a separate document. Any changes made in the course of the evaluation and analysis of these data performed after the locking and un-blinding the database will be documented and fully justified in the final Clinical Study Report (CSR). The contents of the SAP are consistent with the principles described in the ICH E8 and E9 guidelines [1,2].

2 Objectives

Primary:
To evaluate the safety of up to 28 days of SFX-01 dosed at up to 92 mg Sulforaphane (SFN) per day.
To investigate the pharmacokinetic properties of SFN in cerebrospinal fluid (CSF).
To determine if a minimum of 7 days treatment with SFX-01 reduces Middle Cerebral Artery (MCA) peak flow velocity following Subarachnoid Haemorrhage (SAH).

Secondary:

To determine if a minimum of 7 days treatment with SFX-01 improves clinical outcome following SAH as measured using the modified Rankin Scale assessed at 7 days, discharge, 28, 90 and 180 days post ictus.
To determine blood SFN levels (and its metabolites) with treatment with SFX-01 (300mg bid).
To determine CSF SFN levels and kinetics with treatment with SFX-01 (300mg bid).
To determine if up to 28 days treatment with SFX-01 increases blood haptoglobin (HP) levels and decreases malondialdehyde (MDA) levels following SAH.
To determine if up to 28 days treatment with SFX-01 can reduce the incidence of Delayed Cerebral Ischaemia (DCI) following SAH.
To determine if up to 28 days treatment with SFX-01 improves long-term outcome in subjects following SAH.
To determine if up to 28 days of treatment with SFX-01 can reduce iron deposition and cortical atrophy following SAH.

3 Study Design

The study is a randomised, double-blind, parallel-group design comparing SFX-01 (300 mg bd) administered for up to 28 days versus Placebo control in 90 patients who meet the per-protocol criteria for efficacy analyses, who have had a SAH and are referred to neurosurgical units for tertiary care.

Treatment is taken twice-daily either orally or via nasogastric tube (NG). For the first 20 patients, treatment duration is determined by the length of time spent in tertiary care (up to Day 28 post ictus). Following a DSMB review (as per protocol) for all subsequent patients the intended treatment duration is 28 days regardless of whether they remain in tertiary care for the full 28-day period. After treatment all patients are then followed up at discharge from the neurosurgical unit and then subsequently on Day 28, Day 90 and Day 180 post ictus.

A separate and more detailed investigation (sub-study) of the pharmacokinetic properties of SFN parent drug, and the primary metabolite, both in CSF and blood is also undertaken. This will involve up to 12 patients who have an External Ventricular Drain (EVD) fitted as part of their normal standard of care and is undertaken on two separate occasions within the first 7 days post ictus.

The protocol was amended part way through recruitment to stratify the randomisation by WFNS score (1-3 v 4-5) and centre.

3.1 Sample Size

Following protocol amendment 5, the sample size was increased from 90 to up to 120 patients in order to provide 90 who would meet the per protocol criteria and be evaluable for the primary efficacy analysis. Furthermore, this protocol amendment also specified that patients who had potentially received insufficient or incorrect study medication may be replaced.

If the standard deviation is approximately half (53%) of the mean difference in the maximum MCA flow velocity, 90 evaluable patients will have 80% power to detect a statistically significant difference between treatment arms using a 1-sided type 1 error rate of 5%. Statistical significance will be declared if the 2-sided p-value is <0.05, in this case the trial will have >80% power with 90 evaluable patients if the standard deviation is <59% of the mean difference.

4 Analysis Populations

Data from this study will be analysed on two occasions although efficacy endpoints will only be analysed on one occasion. The data cut-off for the first analysis of the study will be 28 days after the last patient is randomized. After this first data-cut-off, the TCD data will be analysed and available safety and PK data will be reported. The second data cut-off will occur 6 months after the last patient is randomized. This second and final analysis will include all available safety, pharmacokinetic and secondary efficacy endpoint data.

4.1 Population Definitions

Four patient populations will provide the basis for all statistical analyses and data presentations.

Per Protocol (PP) population: This will consist of all patients who receive at least 10 doses of randomised treatment within the first 7 days of the first dose of study medication. However, there were nine patients (014, 015, 016, 017, 020, 021, 032, 033, 034) who were either known to have had (n=2), potentially had (n=3), or were associated with patients who had (n=4), a discrepancy in dispensing of randomised therapy, these patients will be excluded from the PP population.

Intent-To-Treat (ITT) population: All randomised patients who receive at least one dose of study medication. This population will include the nine patients excluded from the PP population due to possible errors in dispensing.

The primary population for efficacy endpoints will be the PP population with select efficacy endpoints also analysed in the ITT population (see Section 6.2)

Safety population: All randomised patients who have taken at least one dose of study medication including the nine patients excluded from the PP population due to possible errors in dispensing. This population will be applied to all safety endpoints and pharmacokinetic data recorded in all patients. The safety population will be identical to the ITT population if all patients dosed have efficacy data recorded.

PK Sub-Study population: A group of up to 12 patients fitted with an EVD as part of their normal treatment are to be selected for the pharmacokinetic sub-study.

Given the early stage of development, the PP population is of primary interest and all patients will be analysed according to the treatment they actually receive in all populations. For the ITT population, if due to dispending errors it is known patients received a mixture of SFX-01 and placebo capsules, they will be assigned to the arm for which they received medication for the majority of time.

In addition, clinically important protocol violations will be identified in a blinded manner and then summarised and listed in the clinical study report (CSR).

5 Primary and Secondary Variables

5.1 Trans-Cranial Doppler

An initial TCD reading is taken within 48 hours of ictus. Subsequent TCD readings are then taken three times a week on alternate days (according to standard care procedures). They are performed at least until Day 7 post ictus (± 1)) and then until no longer clinically indicated. Table 1 describes the data collected at each timepoint noting that the individual value recorded represents the maximum of the mean flow recorded during the recording period.

Table 1. TCD Recordings

Reading	Units/Response
Left Middle Cerebral Artery Mean Flow Velocity	cm/s
Right Middle Cerebral Artery Mean Flow Velocity	cm/s
Left Internal Cerebral Artery Mean Flow Velocity	cm/s
Right Internal Cerebral Artery Mean Flow Velocity	cm/s
Assessment Performed	Pre/Post
Lindergaard Ratio Left	none
Lindergaard Ratio Right	none

The primary endpoint is defined as the maximum of all left middle cerebral artery mean flow velocities (MCA-MFV) and right MCA-MFV values recorded after the first dose of randomised therapy including any taken after Day 7.

Baseline values will be defined as:

- The lowest of the left and right MCA-MFV values recorded at the earliest TCD assessment made within 2 days of first dose
- If a patient does not have a TCD recorded within 2 days of dosing, a predicted baseline value will be imputed based on their age, WFNS score (1 to 5), a past medical history of hypertension (yes/no), surgical procedure (clipping, coiling, none), log-CRP and centre as described below.

Amongst patients with baseline values recorded within 2 days of dosing, their baseline value will be regressed on age, WFNS score, hypertension, surgical procedure, log-CRP and centre. The imputed value for patients with a missing baseline value will equal $\mu + \sum x_{ij}\beta_j$ where x_{ij} represent the observed values of the j covariate levels for the i^{th} patient and β_j the corresponding parameter estimates from the regression amongst patients with observed baseline values.

Supplementary analyses will also be performed on the mean of the three largest right or left MCA-MFV recorded at any timepoint after dosing. Additionally, an analysis will be performed to describe the effect over time with timepoints grouped, relative to date of Ictus, as Days 3-4, 5-6, 7-9, 10-14, 15-21, 22-28. Within each timepoint the largest MCA-MFV value will be included in the analysis for each patient. For these two analyses, the same baseline as the primary analysis will be used. Using the timepoint analysis the mean effect of Days 5 to 9 will also be estimated.

The analysis of Lindergaard ratio will mirror the primary analysis of MCA-MFV values: the maximum of all ratios recorded after the first dose of randomised therapy will be analysed and

baseline will be defined as the lowest of the left and right ratios recorded at the earliest TCD recording made within 2 days of first dose, imputing data using the same model approach if baseline values are otherwise missing.

All TCD endpoints will be $\log_e$-transformed prior to analysis.

5.2 Modified Rankin Score

The Modified Rankin Scale (mRS) is recorded at Day 7, 28, 90 and 180 as well as at discharge. mRS will be analysed as a score ranging from 0-6 as displayed in Table 2. Any patient who has died before the respective timepoint will be given a score of 6. Any mRS recorded at the discharge visit that is contained within the protocolled defined time-windows and is closer to the nominal time than any other recordings will be used at that timepoint.

Table 2. Modified Rankin Score (mRS)

mRS recorded response	Score assigned for analysis
No symptom at all	0
No significant disability despite symptoms; able to carry out all usual duties and activities	1
Slight disability; unable to carry out all previous activities, but able to look after own affairs without assistance	2
Moderate disability; requiring some help, but able to walk without assistance	3
Moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance	4
Severe disability; bedridden, incontinent and requiring constant nursing care and attention	5
Dead	6

5.3 Glasgow Outcome Score Extended

The Glasgow Outcome Scale – Extended (GOSE) is recorded at Day 28, 90 and 180. GOSE will be analysed as a score ranging from 1-8 as displayed in Table 3. Any patient who has died before the respective timepoint will be given a score of 1.

Table 3. Glasgow Outcome Scale – Extended (GOSE)

GOSE recorded response	Score assigned for analysis
Death	1
Vegetative state	2
Lower severe disability	3
Upper severe disability	4
Lower moderate disability	5
Upper moderate disability	6
Lower good recovery	7
Upper good recovery	8

5.4 Incidence of Delayed Cerebral Ischaemia

Delayed Cerebral Ischaemia (DCI) is defined as a new focal deficit or reduction in Glasgow Coma Scale ≥2 if not explained by other causes (i.e. re-bleed, hydrocephalus, seizure, meningitis, sepsis or hyponatremia). Patients with the following MedDRA terms will be examined as part of a blinded medical review to decide whether the patient has had a DCI satisfying the outlined criteria:

Delayed ischaemic neurological deficit
Cerebral ischaemia

It is possible additional patients will be identified in a blinded fashion based on other terms.

5.5 Incidence of New Cerebral Infarct

Patients with the following MedDRA terms will be examined as part of a blinded medical review to determine whether the patient experienced a new cerebral infarct on Computed Tomography (CT) or Magnetic Resonance Imaging (MRI):

Cerebral infarction
Haemorrhagic cerebral infarction
Thrombotic cerebral infarction
Haemorrhagic stroke
Ischaemic stroke
Thrombotic stroke
Basal ganglia stroke
Brain stem stroke
Cerebral thrombosis

It is possible additional patients will be identified in a blinded fashion based on other terms.

5.6 Triple H therapy

The dedicated Triple H Therapy for Cerebral Vasospasm CRF will be used to determine for each patient whether such therapy was received.

5.7 SF-36 Quality of Life Survey

The SF-36 Quality of Life Survey is recorded at Day 28, 90 and 180. There are 36 questions in the SF-36 survey each of which are recoded into a value between 0 and 100 as presented in Table 4[3] where 100 represents the best outcome.

Table 4. SF36 recoding individual responses

Item Numbers	Recorded Response Category	Recoded Score
1, 2, 20, 22, 34, 36	1 →	100

	2 →	75
	3 →	50
	4 →	25
	5 →	0
3, 4, 5, 6, 7, 8, 9, 10, 11, 12	1 →	0
	2 →	50
	3 →	100
13, 14, 15, 16, 17, 18, 19	1 →	0
	2 →	100
21, 23, 26, 27, 30	1 →	100
	2 →	80
	3 →	60
	4 →	40
	5 →	20
	6 →	0
24, 25, 28, 29, 31	1 →	0
	2 →	20
	3 →	40
	4 →	60
	5 →	80
	6 →	100
32, 33, 35	1 →	0
	2 →	25
	3 →	50
	4 →	75
	5 →	100

Using these recoded scores, the Physical Health and Mental Health Score will be derived as follows[4]:

1. 35 of the 36 items will be assigned to one of eight subscales as defined in Table 5 and the mean of the recoded scores, $\bar{X}_i$ (i=1 to 8), calculated within each subscale, noting that Item 2 is not assigned to a subscale.
2. A Z score for each subscale, Z_i (i=1 to 8), will then be derived as
$$Z_i = \frac{\bar{X}_i - x_i}{sd_i}$$
where x_i and sd_i are the population reference means and standard deviations for a healthy population. Table 6 displays the appropriate reference means.
3. Each Z_i is multiplied by the corresponding Physical Health coefficient and summed to give an overall Physical Health Sum. The same approach is applied to the Mental Health coefficients to give an overall Mental Health Sum
4. The Physical Health and Mental Health Scores are then calculated by multiplying the respective Physical Health and Mental Health Sum by 10 and adding 50 to the product. A patient with responses typical of a general population will have a score of 50 and perfect health would correspond to Physical Health and Mental Health Scores of 57.87 and 62.14 respectively.

Whilst reference means for a US population are to be applied it has been shown that their application to a UK population gives nearly identical results for the Physical Health and Mental Health Score compared to the use of UK specific reference means[5].

Any patient who has died before the respective timepoint will be assigned a 0 recoded score for each item of the SF-36 prior to deriving the Physical Health and Mental Health Scores.

If less than 50% of the SF-36 questions are completed at a given timepoint or no responses are completed for an individual subscale, the SF-36 data will not be included in the analysis for that patient at that timepoint. If ≥50% of the SF-36 questions are completed, the mean of the observed individual item recorded scores within the respective subscales will be applied to the algorithm.

Table 5. SF36 subscale items

Subscale	Number of Items	Item Numbers included
Physical functioning (PF)	10	3 4 5 6 7 8 9 10 11 12
Role limitations due to physical health (RP)	4	13 14 15 16
Role limitations due to emotional problems (RE)	3	17 18 19
Energy/fatigue (VT)	4	23 27 29 31
Emotional well-being (EW)	5	24 25 26 28 30
Social functioning (SF)	2	20 32
Pain (BP)	2	21 22
General health (GH)	5	1 33 34 35 36

Table 6. SF-36 Reference Values and Physical and Mental Health Coefficients

Subscale	Mean	SD	PH Coefficient	MH Coefficient
PF	84.52	22.89	0.42402	-0.22999
RP	81.20	33.80	0.35119	-0.12329
RE	81.29	33.03	-0.19206	0.43407
VT	61.05	20.87	0.02877	0.23534
EW	74.84	18.01	-0.22069	0.48581
SF	83.60	22.38	-0.00753	0.26876
BP	75.49	23.56	0.31754	-0.09731
GH	72.21	20.17	0.24954	-0.01571

PH = Physical Health, MH=Mental Health

5.8 CLCE-24

The Checklist for Cognitive and Emotional Consequences (CLCE-24) is recorded at 90 and 180 days. The CLCE-24 instrument consists of 24 questions with possible responses of 'No', 'Yes, but not severe', 'Yes, severely hindering daily life' and 'I am not sure'. The problems are split into cognitive problems, corresponding to the first 13 questions, and emotional problems, corresponding to the next 9 questions. The final 2 questions solicit information on

other specified problems; these will not be used in the analysis. Data will be analysed separately for emotional and cognitive problems. The number of questions, within each subscale, that has a response of either 'Yes, severely hindering daily life' or 'Yes, but not severe' will be calculated and this number will be recoded to a CLCE score as described in Table 7. The CLCE-24 score will be subject to statistical analysis.

If a patient dies before a given timepoint they will be assigned a score of 3. If less than 50% of the CLCE-24 questions are completed at a given timepoint the data will not be included in the analysis for that patient at that timepoint. Otherwise, the percentage of responses will be calculated using 24 as the denominator. In determining evaluability, a response of 'I am not sure' will be considered a completed response.

Table 7. CLCE-24 Score

Percentage of responses recorded as 'Yes, severely hindering daily life' or 'Yes, but not severe'	Score assigned for analysis
0%	0
0% < to < 25%	1
25% ≤ to < 50%	2
≥50%	3

5.9 BICRO-39

The Brain Injury Community Rehabilitation Outcomes Scale (BICRO-39) is recorded at 90 and 180 days and consists of 39 questions scored from 0= the most favourable response (no help or prompts, once a week or more, most or all days, several hours a day, more than 20 hours a week, never - as appropriate) to 5=the least favourable response (don't do at all, not applicable or never, almost always – as appropriate). The BICRO-39 is divided into 8 subscales as presented in Table 8. The subscale score is calculated as the mean of the individual scores within that subscale.

An overall score will also be calculated as the mean score across the 34 individual scores that are not included in the contact with partner/children and contact with parents/siblings subscales. These scales are excluded as the desirable direction may vary between patients; some patients may wish to be less reliant on carers if they are relatives and in others alienation from family member maybe a problem[6].

Any patient who has died before the respective timepoint will be assigned a subscale and an overall score of 5 corresponding to the worst outcome. If less than 50% of the 39 questions are completed at a given timepoint, or no questions have been answered in one of the 6 subscales contributing to the overall score, none of the BICRO-39 data will be included in the analysis at that timepoint. Otherwise, if ≥50% but less than 100% of the BICRO-39 questions are completed, the mean of the recorded scores within the respective subscales will be used in the analysis of subscales. However in this case, the overall score will be calculated as a weighted average of the individual subscale scores with weights of 6/34 for all subscales included apart from productive employment which receives a weight of 4/34. This approach ensures that if missing data predominates in one subscale its contribution is not down-weighted in the overall score.

Table 8. BICRO subscales

Subscale	Number of items
Personal care	6
Mobility	6
Self-organisation	6
Contact with partner/children	2
Contact with parents/siblings	3
Socialising	6
Productive employment	4
Psychological well-being	6

5.10 Subarachnoid Haemorrhage Outcomes Tool

The Subarachnoid Haemorrhage Outcomes Tool (SAHOT) is recorded at Day 28, 90 and 180. days and consists of 56 questions scored 0 for 'No change', 1 for 'Some change' and 2 for 'Large or severe change'. The only possible exception is the 'Quality of relationship with those closest' question, which, if the subsequent question indicates the relationship is better, will be scored as 0 regardless of the response.

The sum of the scores across all 56 questions is calculated to give a raw total score and depending on the outcome corresponds to a SAHOT category[7] (Table 9), which be analysed statistically. If the patient has died prior to the timepoint they are assigned to the worst category of 9.

Table 9. SAHOT categories

Raw score	SAHOT category
0–7	1 (best outcome)
8–17	2
18–29	3
30–42	4
43–56	5
57–73	6
74–89	7
90–112	8
Not applicable	9 (death)

If less than 50% of the 56 questions are either not completed or no questions are completed in a subscale, the data will not be included in the analysis for that patient at that timepoint. Otherwise, the raw score will be scaled up prior to assigning the SAHOT category as follows:

$$\textit{Scaled raw score} = \sum_{j=1}^{3} \frac{1}{p_j} \sum_{i=1}^{n_j} x_i$$

where x_i represents the individual question score for the n_j recorded answers in the j^{th} subscale and p_j represents the proportion of intended questions answered for the j^{th} subscale.

This approach will ensure that if missing data predominates in one subscale it is not under-represented in the scaled raw score. A response of 'NA' will count as a completed response.

5.11 Length of acute hospital stay

The length of the acute hospital stay will be defined as the date of discharge (as recorded on the DISCHARG CRF) – date of ictus+1. Patients who die prior to discharge from tertiary care will be censored one day longer than the longest observed stay in either treatment group. If any patient has yet to be discharged at the time of the data cut-off they will be censored at the data cut-off date minus date of ictus+1.

A sensitivity analysis will be performed by setting the censored date of discharge at the date of death for patients who die prior to discharge.

Discharge location will be summarised only and not subject to formal statistical analysis.

5.12 MRI Susceptibility Weighted Imaging

These data will be summarised separately from the CSR.

5.13 Safety Data

Recording of AEs starts from informed consent and continues until at least 30 days post last dose. The definition of AEs and SAEs are given in the protocol.

Adverse events starting on or after the first treatment dose are therefore defined as Treatment Emergent Adverse Events (TEAEs). Any AE that starts more than 30 days past last dose and is considered to be at least possibly related to study treatment should also be defined as a TEAE. Any event starting before the first dose of study drug is identified as a Pre-Treatment Adverse Event (PTAE. Any event starting before the first dose of study drug that subsequently worsens is also be defined as a TEAE.

All events are coded using the MedDRA dictionary. The actual description of the AE is matched, as closely as possible, with the Lower Level Term in the dictionary.

All AEs are classified as described in Table 10.

Table 10. Classification of AEs

AE Classification	**AE Categories**
Severity	Mild/Moderate/Severe/Life threatening
Serious	Yes / No
Relationship	Certain/Probable/Possible/Unlikely
Acton taken	Permanently discontinued/Stopped temporarily/Dose reduced/ Dose increased/ No action taken/ Unknown
Outcome	Recovered or resolved / Recovering or resolving / Not recovering or resolved / Recovered or resolved with sequelae / Fatal / Unknown

Drug related events will be defined as ones whose relationship is given as certain, probable or possible. Any events with missing relationship to study drug will be tabulated as related. Any events with missing intensity will be tabulated in a separate category. If a patient has an AE which changes in severity this will be recorded as a separate occurrence of the same event. Serious adverse events (SAEs) are classified as described in the protocol. If a patient has more than one occurrence of the same AE and at least one is considered drug-related, the patient will be included as having a drug-related AE for that AE preferred term. Similarly, if a patient has more than one occurrence of the same AE and they have different severities, they will be summarised at the worst severity for that AE preferred term.

When summarising the time to onset of an AE the day of first dose will be used throughout as a reference point. If only a month is provided for date of onset, a conservative approach will be followed and events will be assumed to start at max(date of first dose, first day of the month). Likewise, if only a month is provided for the end date, the event will be assumed to have resolved at min(last date of month, date of onset for a new occurrence of the same AE for that patient).

For laboratory data all numeric blood results are classified by the investigator as Clinically Significant / Not Clinically Significant. All categorical urine results are classified as Clinically Significant (Yes / No). All blood and urine samples taken for safety evaluations are assayed by the local laboratory at each hospital. Data will be pooled across centres and summarised by treatment arm despite any differences in reference ranges.

5.14 Pharmacokinetic Data

No derived parameters for CSF and blood HP and MDA concentrations will be created. The method of analysis and presentation is described in a later section. This also applies to Sulforaphane (SFN) and SFN metabolite concentrations at Day 7 from patients who do not take part in the sub-study.

For patients entering the sub-study, SFN parent drug and metabolite concentrations are measured serially in both CSF and blood samples obtained pre-dosing and then hourly for up to 6 hours in a subset of 12 EVD patients on one of the first 3 doses and at Day 7. PK parameters for both CSF and blood will be derived for each individual patient using the concentrations over time profiles, data permitting. Full details of the pharmacokinetic analyses will be provided separately.

5.15 Time-Windows

The protocolled defined visit windows for assessments are +/-1 for Day 7, -2 at Discharge, -6/+2 at Day 28, +/-14 at Day 90 and +/-28 at Day 180. However, for the purpose of any timepoint analyses, data will be included at the visit closest to the time the recordings were taken.

6 Statistical Methods

6.1 General Considerations

All data will be described and analysed according to treatment arm (SFX-01, Placebo) and day and time of assessment (if appropriate), for each patient population (PP, ITT, Safety, sub-study PK).

A (two-sided) significance level of 5% (<0.05) will be implemented throughout. The null hypothesis assumed throughout is that there is no difference between the active treatment and the control for any comparison performed (SFX-01 vs Placebo).

Part way through the trial the randomization was amended to stratify by WFNS score (1-3 v 4-5) and centre. For purposes of analyses that are stratified, WFNS score will be taken from the CRFs for patients randomized prior to the amendment and from the random scheme for patients randomized after the amendment.

6.2 Analysis Methods

Each endpoint subject to formal statistical analysis is listed in Table 11. To investigate the robustness of the primary analysis of each endpoint additional analyses will be performed. These are described as sensitivity analyses if they assess whether the results are influenced by departures from assumptions made in the primary analysis and are described as supplementary analyses if they assess a closely related measure not considered as important as the one chosen in the primary analysis.

Table 11. Formal statistical analyses to be conducted and pre-planned supplementary and sensitivity analyses

Endpoint	Notes
TCD	Primary: maximum post-dose MCA mean flow velocity Sensitivity analysis: • Removing baseline data from the analysis model if it appears SFX-01 has had an effect by Day2 Supplementary analyses: • Mean of 3 largest post-dose MCA mean flow velocities • Maximum post-dose Lindergaard ratio • Maximum MCA mean flow velocity per timepoint • Mean effect over days 5 to 9 of maximum MCA mean flow velocity.
Modified Rankin Scale	Analysis at Day 7, 28, 90 and 180 and at discharge Sensitivity analysis: • If more than 15% of patients have missing data at Day 180 in either treatment arm, the analysis at each timepoint will be repeated by carrying forward the

	outcome from the most recent timepoint with recorded data..
Glasgow Outcome Score - Extended	Analysis at Day 28, 90 and 180 Sensitivity analysis: • If more than 15% of patients have missing data at Day 180 in either treatment arm, , the analysis at each timepoint will be repeated by carrying forward the outcome from the most recent timepoint with recorded data..
Delayed Cerebral Ischaemia	Proportion of patients with an event
New Cerebral Infarct	Proportion of patients with an event
Triple H Therapy	Proportion of patients with hypertensive (Triple H) therapy
SF-36	Physical and Mental Health scores at Day 28, 90 and 180. Sensitivity analysis: • If more than 15% of patients have missing data at Day 180 in either treatment arm, missing data diagnostics performed.
CLCE-24	Score based on the percentage of Cognitive and Emotional questions at Day 90 and 180 that indicate problems that severely hinder daily life Sensitivity analysis: • If more than 15% of patients have missing data at Day 180 in either treatment arm, missing data diagnostics performed.
BICRO-39	Primary: overall BICRO score at Day 90 and 180 Supplementary analyses: • 8 subscale scores at Day 90 and 180 Sensitivity analysis: • If more than 15% of patients have missing data at Day 180 in either treatment arm, missing data diagnostics performed.
SAHOT	SAHOT category at Day 28, 90 and 180 Sensitivity analysis: • If more than 15% of patients have missing data at Day 180 in either treatment arm, missing data diagnostics performed
Length of Acute Hospital Stay	Primary: including patients who die prior to discharge censoring them 1 day longer than the largest observed stay Sensitivity analysis; • Censoring at the date of death as end of hospital stay for patients who die prior to discharge
Blood & CSF HP and MDA	Blood concentrations of HP & MDA at Day 7 and D28 CSF concentrations of HP & MDA at Day 7

Analyses of the TCD, mRS and GOSE endpoints will be performed in both the PP and ITT populations with the PP population considered as primary. Other endpoints will initially only be analysed in the PP population and will only be analysed in the ITT population if there is a meaningful discrepancy in the results of endpoints analysed in both populations.

6.3 Multiplicity

The primary endpoint is the maximum post-dose MCA mean flow velocity.

Whilst no formal multiplicity adjustments will be made amongst the secondary endpoints, the two key secondary endpoints are:
1) Modified Rankin Score at 90 days
2) GOSE score at 90 days

Individual BICRO subscales will not be considered statistically significant unless the overall BICRO score is also statistically significant at the same timepoint.

The TCD data will analysed in advance of the other secondary endpoints. However, given that each endpoint will be analysed only once no adjustments will be made to the significance levels for each endpoint.

6.4 Analysis of the Primary Endpoint

All TCD endpoints will be $\log_e$-transformed prior to analysis including baseline values. The primary endpoint of maximum post-dose MCA mean flow velocity will be analysed using an Analysis of Variance (ANOVA) model with terms for treatment, baseline (as defined in Section 5.1), WFNS grade (1-3 v 4-5), hypertension (yes vs no), surgical procedure (clipping vs coiling vs none), log-CRP and age fitted a continuous covariate. The statistical significance of treatment will be based on type III sums of squares. The supplementary analyses of the mean of the 3 largest post-dose MCA mean flow velocities and the maximum post-dose Lindergaard ratio will use the same methods.

The maximum MCA mean flow velocity per timepoint will be analysed using a mixed model repeated measures (MMRM) approach with visits grouped, relative to date of Ictus, as Days 3-4, 5-6, 7-9, 10-14, 15-21, 22-28 and baseline covariates as defined for the primary analysis. Treatment effects for each visit will be obtained from the same model using the corresponding treatment-by-visit estimate. The MMRM will include terms for treatment, visit, treatment-by-visit, baseline-by-visit, covariates and observations blocked by subject. An unstructured covariance matrix will be used to model the within-subject error, along with restricted maximum likelihood and the Kenward-Roger approximation to estimate the degrees of freedom. If the model will not converge with an unstructured covariance matrix, heterogenous Toeplitz and Toeplitz patterns will be tried in that order until the model will converge.

An example of the corresponding SAS code is given below

```
PROC MIXED data=data1 method=reml;
 CLASS trt vis pat wfns htn surg;
 MODEL lnTCD=base trt vis trt*vis base*vis age wfns htn surg lcrp / ddfm= KR;
```

```
 REPEATED vis / type=un subject=pat;
 LSMEANS trt*vis / slice=vis;
RUN;
```

The mean effect over days 5 to 9 will be estimated from the same model using an appropriate ESTIMATE statement weighting each timeperiod equally.

The treatment effect for each analysis will be presented in terms of a ratio (SFX-01:placebo), representing the ratio of the geometric lsmeans (glsmean) for each treatment arm, together with its 95% confidence interval. The glsmean for each arm will be calculated by exponentiating the lsmeans produced from the analysis of the $\log_e$-transformed data.

The assumptions of normality will be assessed by normal probability plots of the residuals and plotting standardized residuals versus predicted values.

Many of the baseline values used in the analysis will have been recorded shortly after the patient has been dosed with randomized treatment but before it is anticipated any treatment effect will merge, will be included. If there is any evidence that SFX-01 has had an effect by Day2 a sensitivity analysis will be performed where the baseline term is removed from the ANOVA model and centre is added to the list of covariates.

6.5 Analysis of Secondary Efficacy Endpoints

The mRS, GOSE, SAHOT and CLCE will be analysed using a proportional odds logistic regression model to each timepoint separately including the following covariates: treatment, age (fitted as continuous variable), WFNS score (fitted as a class variable), hypertension (yes vs no), surgical procedure (clipping vs coiling vs none) and log-CRP. The treatment effect will be described using an odds ratio together with its likelihood ratio based 95% confidence interval. The statistical significance of the treatment effect will be taken from the chi-square test for the difference in deviance when removing treatment from the model. GOSE scores will be reversed prior to analysis so the odds of a better response is being modelled. PROC LOGISTIC can be used to analyse the data. If the model does not converge, any covariates that are not statistically significant will be removed in order from least significant until the model converges. If the model still does not converge, the outcome level with the fewest observations will be combined with the next most severe outcome and this process repeated until convergence is achieved.

BICRO and SF-36 will be analysed using Van-Elteren's test with 4 strata defined by age (above and below the median) and WFNS score (1-3 v 4-5). The treatment effect will be described using an un-stratified Hodges-Lehmann (HL) estimate of median difference together with its 95% confidence interval. The test can be implemented in SAS as follows:

```
proc npar1way data=data1 HL;
 strata strata/wilcoxon;
 class trt;
 var resp;
 run;
```

The proportion of patients with delayed cerebral ischaemia, new cerebral infarct and receiving triple H therapy will be analysed using logistic regression with the same set of covariates used for mRS. The statistical significance of the treatment effect will be taken from the chi-square test for the difference in deviance when removing treatment from the model. The treatment

effect will be described in terms of an odds ratio together with its likelihood ratio based 95% confidence interval.

The length of acute hospital stay will be analysed using a Cox proportional hazards model with the same set of covariates used for mRS. The statistical significance of the treatment effect will be taken from the chi-square test for the difference in deviance when removing treatment from the model. The treatment effect will be described in terms of a hazard ratio together with its profile likelihood 95% confidence interval. In addition, the median will be reported and taken from the 50^{th} percentile of the associated Kaplan-Meier curves.

6.6 Analysis of Pharmacokinetic Endpoints

Blood concentrations, log_e-transformed prior to analysis, of HP & MDA at Day 7 and D28 will be analysed by MMRM using the same model as described in the analysis of TCD data. Both EVD and non-EVD data will be included in the same model. This model will be used to derive separate treatment effects at Day 7 and Day 28. The baseline term in the models will be the blood concentrations recorded between pre-dose and 48h. A separate summary of the baseline concentrations will confirm that no treatment effect has emerged by 48h by summarising data according to whether data were collected pre-dose, within 24h and between 24 and 48h after dosing. If there is evidence of an effect within 48h, the nominal baseline values will be fitted as a separate visit and a covariate added indicating whether the patient had an EVD sited or not.

Log_e-transformed CSF concentrations of HP & MDA at Day 7 will be analysed using an ANCOVA model with a 2-level covariate denoting whether the CSF values were taken from an EVD or lumbar puncture (LP). A treatment-by-covariate (EVD vs LP) interaction will be added to assess whether the effect of SFX-01 differs by whether the CSF was obtained from EVD or LP together with subgroup analyses for EVD and LP patients separately.

In all cases, the treatment effect will be presented as the ratio of glsmeans and associated 95% confidence interval having exponentiated the lsmeans and confidence limits calculated on the log_e-scale.

Other pharmacokinetic measurements will be summarised only.

6.7 Missing Data Diagnostics

For the mRS, GOSE, SAHOT, CLCE, BICRO and SF-36 endpoints, if more than 15% of patients have missing data for the respective endpoint in either treatment arm at Day 180, the possible impact of these data on the primary analyses will be explored as follows:

- For all endpoints the number of patients with missing data will be summarised. In addition, baseline WFNS score (1-3 v 4-5) will be summarized by treatment arm for each endpoint at each timepoint according to whether data were missing at that timepoint.
- For mRS and GOSE only the analysis at each timepoint will be repeated by carrying forward the outcome from the most recent timepoint with recorded data.

The purpose of these analyses is to assess whether the amount of missing data differs by treatment arm and whether patients with missing data initially had a worse prognosis or were performing worse on other measures recorded at the same timepoint. For example, if there

are more patients in the placebo arm with missing data and those that have missing data were tending to respond less well, this would suggest the treatment effect may have been underestimated.

If the results are marginal for a particular endpoint these investigations may be performed despite neither arm having 15% of patients with missing data. Dependent on results further analyses may be performed to assess the possible impact of missing data.

For TCD endpoints it is not anticipated that missing data could have a bearing on the outcome of the results given the proximity of the measurements to dosing.

Only the year of birth has been recorded. When calculating age, a date of birth of 1st July will be assumed.

6.8 Subgroup analyses

Subgroup analyses will be performed for maximum post-dose MCA mean flow velocity, mRS at 90 days and GOSE at 90 days for the following variables: centre, WFNS score (1-3 v 4-5), Fisher grade, time from ictus (<24h, 24-48h, >48h), Age group (≤49, 50-59,≥60), gender, surgical procedure (coiling, clipping, not done), location of aneurysm (anterior, posterior), main vessel of aneurysm (anterior, middle, internal, vertebrobasilar) and whether the patient developed a secondary bleed prior to randomisation. Additionally, a subgroup analysis of the primary TCD endpoint will be performed by baseline value split according to quartiles. Results will not be presented for any subgroup level that contains fewer than 10 patients.

Data will be analysed separately for each level of the subgroup. For the primary TCD endpoint, the ANOVA model will be re-run and the treatment effect and associated 95% confidence intervals presented. For mRS and GOSE at 90 days, the logistic regression model will performed within each level of the subgroup with the odds ratio and associated 95% confidence intervals presented. Results from all subgroups will be presented in a forest plot for each endpoint separately.

If there is evidence of heterogeneity of the treatment effect amongst subgroups the following tests will be performed:

1. A treatment-by-subgroup interaction term will be added to a model containing the terms included in the primary analysis model together with the main effect of the subgroup if not already included in the model. The p-value for the change in model fit by adding the interaction term will be presented
2. A test of the collective heterogeneity amongst subgroups will performed. This will be achieved by comparing a base model including treatment and all main effects of subgroups with a model containing all two-way interactions of treatment and subgroup. The p-value for the change in model fit by adding all of the interaction terms simultaneously will be presented and described as a global interaction test.

Age will be fitted as a continuous covariate in the interaction tests.

6.9 Changes from Protocol

1. The use of modelling rather than hypothesis testing where possible to allow more powerful analysis through inclusion of covariates and the generation of treatment estimates with confidence intervals.
2. Refinement of the per-protocol definition to consist of patients who receive at least 10 doses of randomised treatment within the first 7 days post-ictus and to exclude patients with potential dispensing errors.
3. Clarification that mRS and GOSE at 90 days are key secondary endpoints

7 Presentation of Data

This section describes the presentation of the results, supporting displays will be detailed in a separate document.

General Information

All continuous data will be presented as means, standard deviation, minimum, maximum, median, lower quartile, and upper quartile as well as number of observations for both actual values and change from baseline data. Log_e-transformed data, PK and TCD, will be summarised using geometric mean, coefficient of variation (CV), minimum, maximum, median, lower quartile, and upper quartile as well as number of observations. CV will be calculated as sqrt(exp(sigma^2)-1), where sigma is the standard deviation of the log_e-transformed data. All descriptive summaries will be displayed to one more decimal place than actually measured. All categorical data will be presented in contingency tables as frequencies and percentages and the denominator will be the number of patients available in the relevant population who have data recorded.

All the tables will be summarised using the treatment arm allocation (SFX-01, Placebo), day and time post ictus or dosing as appropriate. Patients will be assigned to time windows as described in Section 5.15. Any assessments not assigned to time-windows, as other measurements are closer, will be flagged in the appropriate supporting listings.

All demographic summaries will be produced for both the ITT and PP populations according to treatment arm (SFX-01, Placebo). Summaries of TCD, mRS and GOSE data will also be produced for both the ITT and PP populations according to treatment arm (SFX-01, Placebo). Other secondary efficacy endpoints will be produced for only the PP population. Safety summaries and pharmacokinetic data reported outside of the sub-study will be produced for the Safety population and non-compartmental PK data from the sub-study will be summarised for the Sub-Study population only.

Data presentations

Disposition
A categorical summary of patient status detailing the number and percentage of: Screened, Randomized, Treated, Died, Completed will be produced by treatment arm. The denominator for percentages will be the number randomized so no percentage will be presented for the Screened row. This summary will be repeated for the patients in the PK sub-study. The number and percentage of subjects in each patient population (PP, ITT, Safety, PK) will also be given as well as the number of patients who have an EVD sited. Amongst patients who did not complete the study, the reason for withdrawal will also be summarised together with a cumulative incidence plot of time to withdrawal by treatment arm produced for both the PP and ITT populations.

Major Protocol Violations And Deviations
Major violations are to be summarised by treatment arm (SFX-01, Placebo) and overall for all patients and will include the nine patients associated with potential dispensing errors. The number of patients not meeting all inclusion and exclusion criteria will be summarised by treatment arm along with the reason. Supporting listings for both violations and deviations will be included which identify patient, treatment arm and the assigned population.

Qualifying Evaluations (Surgery, Eligibility And Screening Procedures)
Dates and times of all qualifying evaluations will be listed as: Onset of Ictus, Surgery (plus WFNS and any Triple H Therapy for Cerebral Vasospasm), Eligibility, Investigator Consent, Randomisation and Dosing. Other reported dates included: Discharge, MRI at follow up and End Of Study (EOS).

The time between Ictus and the patient receiving their first dose of randomized medication will be summarized for both the ITT and PP populations by treatment arm.

Surgery Details
Surgery details will be summarised for the ITT and PP population by treatment arm. Type of scan (CT/MRI), Angiographic assessment performed (CTA/ DSA/MRA), Fisher grading (No haemorrhage, SAH <1mm, SAH>1 mm, SAH any thickness with IV haemorrhage or parenchymal extension) and Surgical procedure (Coiling/Clipping), Triple H therapy for Cerebral Vasospasm (Yes/No) and WFNS (Grading I, II, III, IV, V).

Eligibility And Screening Procedures
Demographic data will be summarised for the ITT and PP population by treatment arm which will include a summary of the data recorded on aneurysm location and whether the patient developed a secondary bleed prior to randomisation. Medical History, Physical Examination, Pregnancy Test (if applicable) and Prior Medications will be summarised for the Safety population by treatment arm.

Concomitant Medications
All medications taken after the start of treatment are summarised according to WHODD ATC categorised through to level 4.

Randomisation, Dosing And Drug Accountability
All patient randomisation details will be listed indicating the treatment randomised and the treatment actually received. Any miss-randomisations will be flagged. In particular, the intended and actual treatment received on each day for the nine patients associated with potential dispensing errors will be listed.

Exposure to study drug will be described by summarising the dosing duration (date of last dose – date of first dose +1), total number of doses given and compliance (100*total number of doses given /[2*dosing duration]/) by treatment arm for both the PP and Safety populations. Individual details will be listed.

TCD
The maximum post-dose MCA mean flow velocity and the corresponding baseline value (see Section 5.1) will be summarised according to treatment arm (SFX-01, Placebo) for both the PP and ITT populations. The number of patients who have data imputed at baseline will also be summarised by treatment arm. In addition, the baseline values will be split according to whether they occurred before dosing, on Day1 and Day 2 and summarised by treatment arm.

Results of the statistical analysis will be presented as described in Section 6.4. A graphical presentation of the treatment effects and lsmeans over time will be produced using the results of the MMRM analysis.

Glasgow Coma Score (GCS)

The GCS daily scores (except Baseline) will be summarised as continuous data for both the both the best and worst daily score and for both the PP and ITT populations. Patients will be grouped into time windows as Days 3-4, 5-6, 7-9, 10-14, 15-21, 22-28.

Secondary Efficacy Endpoints

For mRS, CLCE-24, SAHOT and GOSE the percentage and cumulative percentage (best to worst score) of patients in each category will be summarised by treatment arm. SF-36 and BICRO-39 will be summarised as continuous data by treatment arm. The number and percentage patients with DCI, new cerebral infarct and institution of triple H therapy will be summarised by treatment arm together with a cumulative incidence plot displaying the time to each of these events across patients by treatment arm. A Kaplan-Meier (KM) curve of the length of acute hospital stay, will be presented along with an associated table presenting the 25^{th}, 50^{th} (median) and 75^{th} percentiles derived from the KM curve. Discharge location will be summarised by treatment arm.

The results of the statistical analyses of all secondary endpoints will be presented as described in Section 6.5. Specified missing data diagnostics will also be presented by treatment arm as necessary as described in Section 6.7. Subgroup analyses will be presented as described in Section 6.8.

Pharmacokinetic Endpoints

The results of the analysis of blood HP and MDA concentrations at Day 7 and 28 and CSF HP and MDA concentrations at Day 7 will be presented as described in Section 6.6. The blood and CSF HP and MDA concentrations will be summarised by treatment arm at all timepoints noting that EVD patients have concentrations recorded at extra timepoints. For timepoints in common to both EVD and non-EVD patients data will pooled across groups as well as presenting the data split by the use of an EVD.

For patients in the sub-study, the derived PK parameters in both CSF and blood will be summarised as described for $\log_e$-transformed data except for Tmax where the geometric mean and CV will not be calculated and median presented. Supporting graphical displays of individual and mean profiles will be produced by sampling day.

Individual SFN and SFN metabolite concentrations recorded on Day 7 and any concentrations recorded in the sub-study for patients outside of the sub-study using the summary measures described for $\log_e$-transformed data pooled across EVD and non-EVD groups. If <50% of concentrations are below the lower limit of quantification (LOQ) then data will be summarised substituting the LOQ in the calculation of the geometric mean and CV, otherwise these two parameters will not be presented. The median, LQ and UQ will be displayed as 'NQ' if those statistics correspond to a value below the LOQ. These summaries will only be produced for the SFX-01 group.

Note proteomic and genetic data will not be summarised as part of the CSR.

Adverse Events (AEs)

MedDRA coded events will be summarized by treatment arm and overall. The number of patients reporting adverse events will be presented according to the coded Preferred Term and System Organ Class (SOC).

An overview table of the number and percentage of patients with any Pre-Treatment Adverse Event (PTAE), TEAEs, Deaths, Serious TEAEs, All Serious AEs, TEAEs leading to discontinuation, maximum severity of TEAEs and Drug-Related TEAEs will be presented as a categorical summary according to treatment arm and overall. In the maximum severity row, patients will be counted once according to the worst severity categorized as missing/mild/moderate/severe/life threatening.

The following summarized of AEs will be produced:

- number and percentage of patients with at least one PTAE by Preferred term
- number and percentage of patients with at least one TEAE by Preferred term
- number and percentage of patients with at least one TEAE by Preferred term by maximum severity
- number and percentage of patients with at least one TEAE by Preferred term that is related to study drug
- number and percentage of patients with at least one TEAE by Preferred term that is serious
- number and percentage of patients with at least one adverse event that is serious, to include any serious AEs that occurred prior to dosing with study medication
- number and percentage of patients with at least one TEAE by Preferred term that lead to discontinuation of treatment corresponding to events where action taken with study treatment is 'Permanently discontinued'

In each table events will be grouped according to SOC and within each SOC events will be presented in order of decreasing frequency. The SOC row will present the number of patients with at least one TEAE within that SOC for the subset of events being summarized.

Serious TEAEs will be listed by patient and treatment arm, indicating the name of the AE, PT and SOC, together with details of the start and end dates, event duration (days) and maximum intensity. Additionally, the relation to study drug, action taken, outcome and the date of the first dose of study drug will also be displayed.

Deaths

All deaths during the study will be summarised by treatment arm (from EOS form). Details will also be listings. A KM plot of time to death measured from the date of ictus will be presented by treatment arm. Patients alive will be censored at the visit date completed for the End of Study CRF.

Laboratory Safety Assessments

Safety blood including lipid and coagulation tests performed throughout the study will be summarised as continuous variables by visit and treatment arm. Both actual values and change from baseline will be summarised. In addition, changes from baseline will displayed on a box-plot by visit and treatment arm for each parameter. Clinically significant findings for each parameter will be summarised in a shift-table by treatment arm, where the clinical significance of values recorded within 48h of ictus will determine the row and whether the patient has any later value recorded that is clinically significant will determine the column. Percentages will use the total number of patients in each row as a denominator. Corresponding shift tables will be produced for urine sample parameters.

Data Listings

All data presented in summary tables will be supported by specific referenced data listings. These will identify the subject number, cohort number, treatment arm (SFX-01, placebo) Visit, Dose number, Dose day (if appropriate) and provide actual data or derived data as appropriate.

Listings will be created according to the ICH guidelines, as appropriate for the data collected.

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8 References

1- ICHE9 “Statistical Principles For Clinical Trials” February 1998.

2- ICHE8 “General Considerations For Clinical Trials” July 1997.

3 - ***https://www.rand.org/health/surveys_tools/mos/36-item-short-form/scoring.html***

4 -Taft C, Karlsson J, Sullivan M. Do SF-36 summary component scores accurately summarize subscale scores? Quality of Life Research (2001) 10:395-404

5 -Jenkinson C. Comparison of UK and US methods for weighting and scoring the SF-36 summary measures. J Public Health Medicine (1999) 21:372-376

6 – Powell J, Heslin J, Greenwood R. Community based rehabilitation after severe traumatic brain injury: a randomised controlled trial. J Neurol Neurosurg Psychiatry (2002);72:193–202

7 – Pace A, Mitchell S, Casselden E, Zolnourian A, Glazier J, Foulkes L, Bulters D, Galea I. A subarachnoid haemorrhage-specific outcome tool. Brain (2018); 141:1111-1121

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	1
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4
	2b	Specific objectives or hypotheses	5
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	6
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	Supplement protocol
Participants	4a	Eligibility criteria for participants	6
	4b	Settings and locations where the data were collected	6
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	7
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	7
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	Supplement protocol
	7b	When applicable, explanation of any interim analyses and stopping guidelines	NA
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	7
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	7
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	7
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to	7

		interventions	
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	7
	11b	If relevant, description of the similarity of interventions	7
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	9, Supplement SAP
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	9, Supplement SAP
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	11
	13b	For each group, losses and exclusions after randomisation, together with reasons	11
Recruitment	14a	Dates defining the periods of recruitment and follow-up	11
	14b	Why the trial ended or was stopped	NA
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	11
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	11
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	14
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	14
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	13-14
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	11
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	19
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	20
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	15
Other information			
Registration	23	Registration number and name of trial registry	6
Protocol	24	Where the full trial protocol can be accessed, if available	6

Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	21

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up-to-date references relevant to this checklist, see www.consort-statement.org.

SAS Study Supplemental Figures

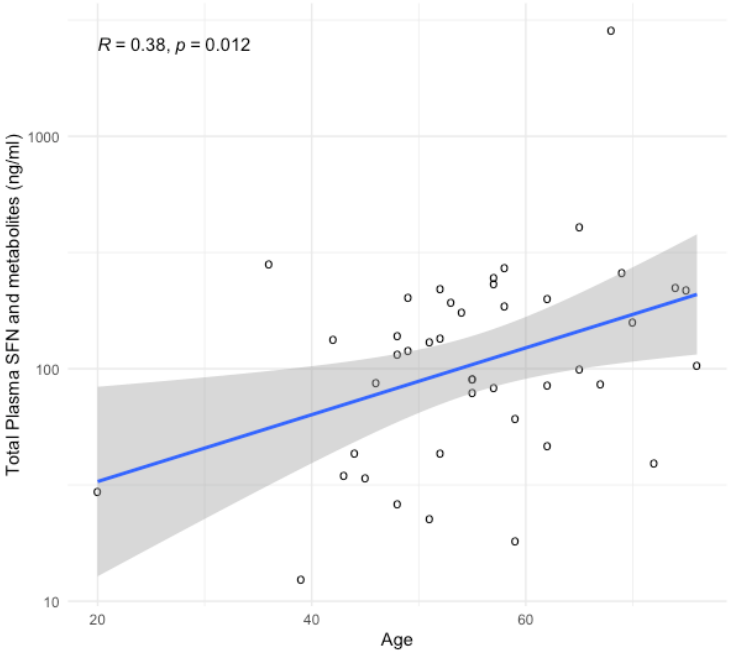

Figure S1. Mean total plasma SFN and metabolites on day 7 vs age. Pearson correlation coefficient (R) and linear regression line with 95% confidence intervals. 43 patients taking SFX-01 per protocol included.

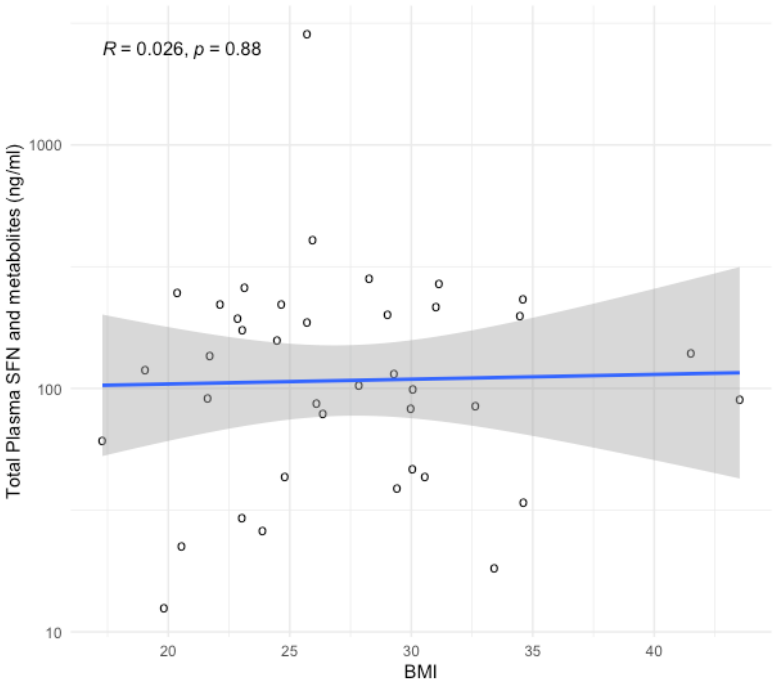

Figure S2 Mean total plasma SFN and metabolites on day 7 vs Body Mass Index (BMI). Pearson correlation coefficient (R) and linear regression line with 95% confidence intervals. 43 patients taking SFX-01 per protocol included.

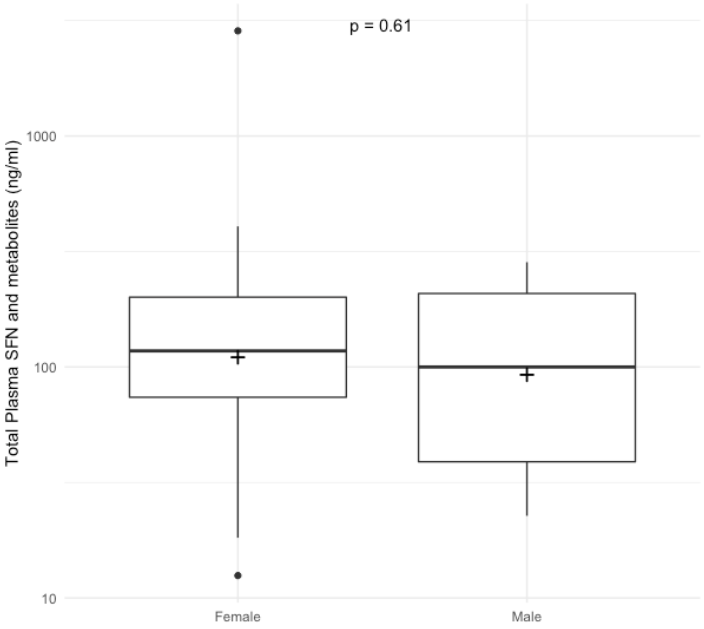

Figure S3 Total Plasma SFN and metabolites (SFN + SFN-GSH + SFN-NAC) for male and female patients. 43 patients taking SFX-01 per protocol included with Plasma samples included. Values below the lower limit of quantification (LLOQ SFN 5mg/ml, SFN-GSH 10mg/ml, SFN-NAC 5mg/ml for both plasma and CSF) are represented as the midpoint of 0 and the LLOQ. P-value on t-test. Means depicted with +.

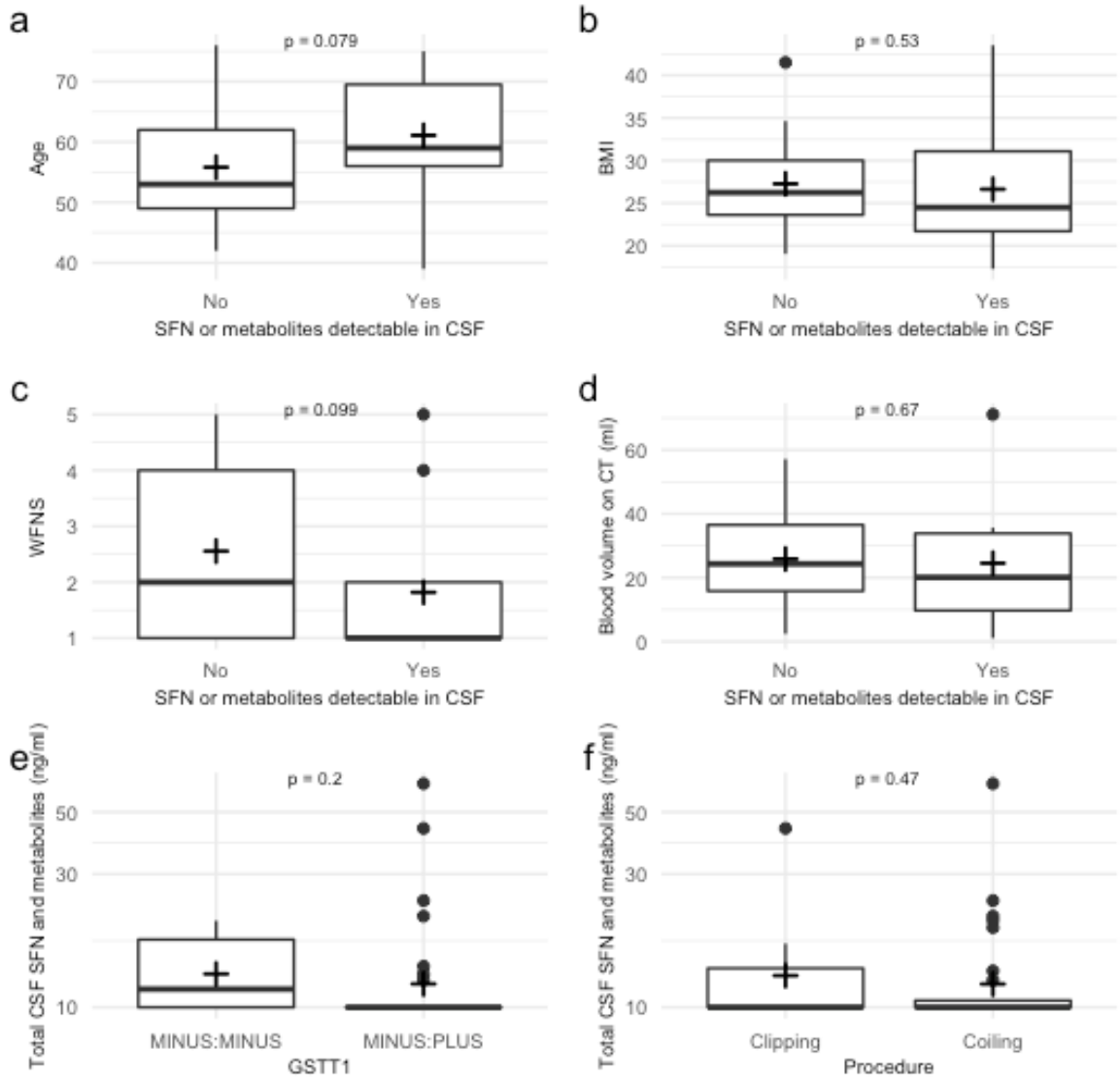

Figure S4 Relationship between baseline variables and detection of SFN or metabolites in CSF in patients receiving SFX01 per protocol.
Blood volume on CT was measured in ml on baseline CT scans performed within 48 hours of ictus and prior to recruitment. Manual segmentation of blood was performed and quantified using MIPAV (Medical Image Processing, Imaging and Visualization) v11.0. The CT image signal intensity threshold was set between 50 and 80 Hounsfield units and converted to a binary mask. Regions of interest representing blood clot were drawn manually on each slice and summed into single 3-dimensional volumes.

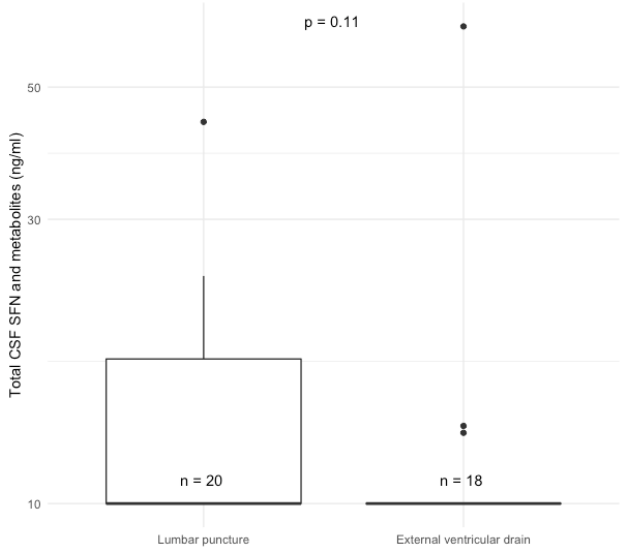

Figure S5 Total CSF SFN and metabolites for samples obtained via lumbar puncture and external ventricular drain on day 7. Patients taking SFX-01 per protocol with CSF samples included. Values below the lower limit of quantification (LLOQ SFN 5mg/ml, SFN-GSH 10mg/ml, SFN-NAC 5mg/ml for both plasma and CSF) are represented as the midpoint of 0 and the LLOQ. There was no significant difference between groups detected (Wilcoxon signed rank W = 224, p = 0.112).

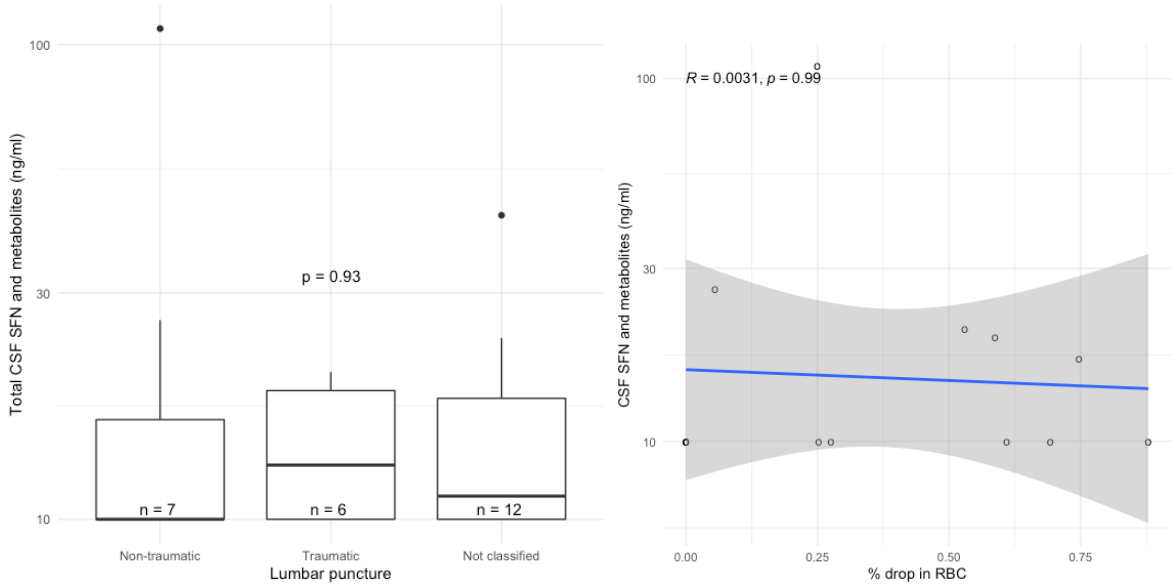

Figure S6 a) Total SFN and metabolites in CSF obtained by lumbar puncture classed by whether it the lumbar puncture was traumatic or not. Non traumatic classed as a drop in red blood cell counts between 1st and 4th bottle of <30% and traumatic as >30% based on Gorchynski et al, and those missing a first or last sample as not classified. There was no significant difference between groups detected (Kruskal-Wallis Chi Square = 3.04, p = 0.219).

b) Relationship between the percentage drop in red blood cells (RBC) between first and last bottle of CSF and total CSF SFN and metabolites. All patients allocated to SFX-01 with CSF samples included and 1st and 4th bottle RBC shown. Spearman correlation coefficient

(R) and linear regression line with 95% confidence intervals. Values below the lower limit of quantification (LLOQ SFN 5mg/ml, SFN-GSH 10mg/ml, SFN-NAC 5mg/ml for both plasma and CSF) are represented as the midpoint of 0 and the LLOQ.

SAS Study Supplemental Methods 2

Sulforaphane sample collection

Plasma was collected in 6.0 mL K_2EDTA collection tubes, containing 0.1 mL of 0.5M citric acid, inverted 8 times, placed on wet ice and centrifuged within 10 minutes at 1500 rcf for 10 min at 4°C before storing at -80. CSF (5mL) was collected in 30 mL universal tubes, containing 0.1 mL of 0.5M citric acid (after discarding the first 3ml if obtained from an EVD). The first 1ml of CSF was used for cell count analysis, and the next 10ml were centrifuged and stored, similar to plasma, for pharmacodynamic analyses.

Sulforaphane quantification

SFN and its metabolites, sulforaphane glutathione (SFN-GSH) and sulforaphane N-acetyl cysteine (SFN-NAC), were quantified by a Good Laboratory Practice (GLP) accredited laboratory (Alderley Analytical) using LC-MS/MS (Waters I-Class UPLC, coupled to a Waters TQ-S Mass Spectrometer) calibrated over 5 to 2000 ng/mL for SFN and SFN-NAC, and 10 to 2000 ng/mL for SFN-GSH. The validation report is in supplementary methods validation report.

Malondialdehyde and Haptoglobin quantification

Malondialdehyde was quantified using a standard colorimetric method (NorthWest Life Science Specialties), which relies on the detection of the reaction product between MDA and thiobarbituric acid, which absorbs strongly at 532 nm.

Haptoglobin was analyzed by rate nephelometry on a Beckman Coulter IMMAGE immunochemistry system, certified to clinical standards. Since haptoglobin determination in CSF requires a lower limit of detection than the Beckman Coulter system provides, an in house validated ultra-performance liquid chromatography (UPLC) assay that measures haptoglobin in the CSF was employed[1]; with this method, haptoglobin concentration in the CSF is expressed in terms of the concentration of haemoglobin it is able to bind.

1. Garland P, Morton MJ, Haskins W, et al. Haemoglobin causes neuronal damage in vivo which is preventable by haptoglobin. *Brain Commun*. 2020;2(1). doi:10.1093/braincomms/fcz053

SAS study Supplemental tables

Treatment emergent adverse events	SFX-300 mg	Placebo
Acute Kidney Injury	3	0
Anaemia	1	3
Arachnoiditis	1	0
Ataxia	1	0
Atrial fibrillation	4	1
Blurred Vision	1	0
Confusion	1	3
Constipation	1	3
Cough	1	0
Delayed Cerebral ischaemia	12	7
Deranged LFTs	46	30
Diarrhoea	2	2
Dry skin	0	1
Dysphagia	1	0
Epistaxis	1	1
Folliculitis	0	1
Hallucinations	2	1
Headache	5	2
Hydrocephalus	10	7
Hypercholesterolaemia	10	14
Hyperglycaemia	1	1
Hyperkalaemia	1	1
Hypernatraemia	6	4
Hypoalbuminaemia	3	3
Hypocalcaemia	2	1
Hypokalaemia	16	13
Hyponatraemia	15	15
Lower respiratory tract infection	22	15
Migraine	1	0
Nausea	9	1
Neutropenia	0	1
Oral thrush	0	1
Photophobia	1	2
Pruritis	0	1
Pulmonary oedema	6	2
Raised fibrinogen	4	6
Raised urea	2	3
Rash	0	2
Retinal artery occlusion	0	1
Seizure	1	2
Tachycardia	2	0
Thrombocytopenia	1	0
Thrombocytosis	2	1
Tinnitus	1	0
Urinary tract infection	10	17
Vasovagal	1	0
Ventriculitis	5	3
Vitreous haemorrhage	7	1
Vomiting	5	1

Table S1 A list of treatment emergent adverse events in the safety population that received at least one dose of SFX-01 or placebo.

	AUC_{last} (h×ng/ml)	C_{max} (ng/ml)	T_{max} (h)
SFN	16.2 (0.725-362)	23.6 (2.12-263)	2.6 (1.52)
SFN-GSH	277 (77.0-995)	118 (35.0-399)	2.5h (1.69)
SFN-NAC	415 (147-1170)	129 (36.7-452)	2.25h (1.16)

Table S2 Day 7 plasma concentrations of SFN, SFN-GSH and SFN-NAC. AUC_{last} geometric mean in h×ng/ml (-sd +sd), C_{max} geometric mean in ng/ml (-sd +sd), and T_{max} arithmetic mean (sd). SFN based on 5 patients with available data.

	SFX-01		Placebo				
Time	n	GLS mean	n	GLS mean	Ratio of means	95% CI	P value
Serum haptoglobin (g/L)							
Baseline	41	1.26	37	1.55			
Day 7	39	2.39	36	2.55	0.937	0.791-1.109	0.446
Day 28	35	1.72	35	1.67	1.031	0.865-1.228	0.731
CSF haptoglobin (mg/L)							
Day 7	40	2.27	37	1.17	1.981	0.992-3.786	0.052
Plasma malondialdehyde (μM)							
Baseline	42	0.40	37	0.37			
Day 7	42	0.34	37	0.38	0.907	0.737-1.116	0.355
Day 28	35	0.30	35	0.31	0.974	0.785-1.209	0.810
CSF malondialdehyde (g/L)							
Day 7	40	0.116	37	0.103	1.123	0.747-1.687	0.572

Table S3. Mixed Models Repeated Measures analysis of serum haptoglobin and plasma malondialdehyde and analysis of variance for CSF haptoglobin and CSF malondialdehyde in the per protocol population. Mixed Models Repeated Measures analysis included terms for treatment, visit, log-baseline, WFNS grade, hypertension, surgical procedure, log-CRP, age, treatment*visit, log-baseline*visit. CSF haptoglobin and malondialdehyde were modelled with an analysis of variance including terms for treatment and CSF source. The geometric least squares (GLS) mean is presented.

SFX-01		Placebo				
n	GLS mean	n	GLS mean	Ratio of means	95% CI	P value
Maximum MCA Flow Velocity at any time						
44	122	41	116	1.046	0.903-1.211	0.545
Maximum Lindegaard ratio at any time						
37	3.29	38	3.11	1.061	0.905-1.243	0.461

Table S4. Analysis of variance of maximum MCA flow velocity and maximum Lindegaard ratio recorded at any time

MCA Flow Velocity							
	SFX-01		Placebo				
Time	n	GLS mean	n	GLS mean	Ratio of means	95% CI	P value
Baseline	44	61.8	40	64.9			
Days 3-4	19	82.3	16	99.0	0.832	0.673-1.028	0.088
Days 5-6	34	97.7	26	100.9	0.968	0.814-1.151	0.711
Days 7-9	38	105.5	40	109.1	0.966	0.824-1.133	0.672
Days 10-14	28	115.9	23	99.3	1.166	0.971-1.400	0.099
Days 15-21	17	90.8	17	86.5	1.050	0.844-1.306	0.662
Days 22-28	7	57.7	7	72.6	0.794	0.577-1.093	0.153

Table S5. Mixed Models Repeated Measures analysis of MCA flow velocity.

SFX-01		Placebo				
n	Events	n	Events (%)	Odds Ratio	95% CI	P value
Delayed Cerebral Ischaemia						
46	9 (19.6%)	44	6 (13.6%)	1.728	0.500-6.463	0.390
Hypertensive therapy						
46	6 (17.4%)	43	7 (16.3%)	0.759	0.218-2.575	0.656

Table S6. Logistic regression analysis of proportion of patients developing delayed cerebral ischaemia and receiving hypertensive therapy.

	SFX-01	Placebo			
Time	n	n	Odds Ratio	95% CI	P value
Day 7	45	43	1.352	0.589-3.134	0.477
Day 28	43	41	1.346	0.583-3.129	0.487
Day 90	43	40	1.598	0.699-3.704	0.268
Day 180	42	38	1.647	0.721-3.821	0.237

Table S7. Proportional odds logistic regression analysis for modified Rankin Score in the per protocol population. Odds ratios >1 favour SFX-01.

	SFX-01	Placebo			
Time	n	n	Odds Ratio	95% CI	P value
Day 28	42	39	0.979	0.422-2.263	0.960
Day 90	39	40	0.917	0.406-2.068	0.834
Day 180	42	38	1.263	0.560-2.868	0.574

Table S8. Proportional odds logistic regression analysis for extended Glasgow Outcome Scores in the per protocol population. GOSE scores were reversed prior to analysis so the odds of a better response was being modelled. Odds ratios >1 favour SFX-01.

SFX-01	Placebo			
n	n	Odds Ratio	95% CI	P value
SF-36 Physical Health				
38	39	-0.539	-4.862-3.784	0.850
36	32	-0.010	-6.606-6.587	0.540
41	35	2.270	-3.566-8.107	0.521
SF-36 Mental Health				
38	39	-2.208	-7.771-3.356	0.319
36	32	-0.440	-7.792-6.912	0.992
41	35	-2.938	-10.228-4.352	0.492

Table S9. Analysis of SF-36 in the per protocol population. SF-36 was analysed using Van-Elteren's test with four strata defined by age (above and below the median) and WFNS score (1-3 v 4-5). The treatment effect was described using an un-stratified Hodges-Lehmann (HL) estimate of median difference together with its 95% confidence interval.

	SFX-01	Placebo			
Time	n	n	Odds Ratio	95% CI	P value
Day 28	42	41	0.667	0.295-1.487	0.323
Day 90	39	34	0.811	0.348-1.882	0.626
Day 180	41	36	1.082	0.464-2.525	0.855

Table S10. Proportional odds logistic regression analysis for the SAHOT in the per protocol population. Odds ratios >1 favour SFX-01.